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CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, AL 35294

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14. ABSTRACT

Bone is a common site of metastasis in breast cancer patients, leading to serious clinical consequences and a poor prognosis. The molecular mechanisms regulating this preferential metastasis of breast cancer to bone have not yet been fully elucidated. I found that CD68 is highly expressed in certain breast cancer cell lines and demonstrated that expression is correlative with bone adhesion ability. I hypothesized that CD68 mediates attachment of breast cancer cells onto bone matrix, subsequently regulating bone metastasis. Using stable shRNA (short hairpin RNA) to knock down CD68 expression in breast cancer cells and forcing expression of CD68 in breast cancer cell lines that do not endogenously express it, I now believe CD68 is not a critical adhesion molecule. However, because CD68 is a lysosomal protein, it may be involved in cell viability. Preliminary work shows a potential for CD68 to be involved in chemoresistance. Additionally, studies may further provide more direct and convincing data. Furthermore, I investigated the role of IL-11 in breast cancer and found that it sustains the pool of osteoclast progenitor cells, which could contribute to osteolysis in breast cancer bone metastasis.

15. SUBJECT TERMS
Breast Cancer Bone Metastasis, CD68, Bone Adhesion

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Introduction

Bone is a common site of metastasis in breast cancer patients, leading to serious clinical consequences and a poor prognosis. The molecular mechanisms regulating this preferential metastasis of breast cancer to bone have not yet been fully elucidated. Using a Phage Display-based approach by biopanning with bone slices, our lab identified macrosialin as a potential mediator of osteoclast adhesion to bone. We hypothesized that CD68 mediates attachment of breast cancer cells onto bone matrix, subsequently regulating bone metastasis. My work shows that expression of CD68 is higher in bone metastatic cell lines of breast carcinoma and that this correlates with the ability to adhere to the bone. However, assays failed to reveal a direct role of CD68 in breast cancer adhesion to bone. Preliminary work shows a potential role of CD68 as a viability factor for breast cancer, specifically with chemoresistance activity. Future studies are needed to further characterize CD68's exact role in breast cancer, which may lead to potential therapeutic strategies. The use of CD68 as a macrophage marker in breast cancer tumors needs to be reevaluated as well, because we have shown that the actual breast cancer cells can express CD68.

Body

Specific Aim 1: Further characterize the role of CD68 in breast cancer cell attachment on bone in vitro

a. Develop stable long-term down-regulation of CD68 by siRNA in breast cancer cell lines MDA-MB-231 and MDA-MB-435. Examine if siRNA down-regulation of CD68 leads to reduced capacity to attach onto bone (Months 1-12)

I first addressed this issue by comparing expression of CD68 in four breast cancer cell lines: MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-468. At both the mRNA (**Figure 1**) and the protein (**Figure 2**) level, CD68 expression was relatively higher in MDA-MB-231 and MDA-MB-435, cell lines known to be bone metastatic as compared with non-bone-lytic lines MDA-MB-468 and MCF-7. Furthermore, in MDA-MN-231 and MDA-MB-435, CD68 expression seems to be orimarily intracellular, as shown by flow cytometry data (**Figure 3**). However, CD68 is known in macrophages to be a rapidly cycled molecule, thus it still has potential as a surface acting molecule. I next addressed the cell adhesion abilities of the breast cancer lines and found that CD68 levels correlate with attachment of breast cancer cells onto bone *in vitro* (**Figure 4**). Breast cancer cells lines were cultured to 90% confluency and then lifted using 2mM EDTA. In order to determine the optimal parameters of the bone adhesion assay, four different timepoints were chosen and cells were cultured under three different media conditions (DMEM only, DMEM with complete levels of serum, and PBS). After incubation, cells were lysed for luciferase activity to determine the number of cells able to attach to the bone experiments had an n-value of 3, and were repeated two times.

In the original proposal, I showed that CD68 antibody blocked interaction between bone and MDA-MB-231 breast cancer cell lines, but MDA-MB-435. I repeated the CD68- antibody adhesion studies to determine whether the initial results were affected by the adhesion assays themselves. I found that any antibody treatment might be confounding when used in bone adhesion assay (**Figure 5**). In order to look more specifically at this interaction, successful stable knockdown of CD68 in breast cancer cell lines MDA-MB-435 and MDA-MB-231 was achieved using shRNA. Corresponding control cells were also made using scrambled shRNA. These cells were then incubated on bovine bone slices for 1 hour, as determined by the timepoint experiment in figure 4, and then lysed for luciferase activity to determine the number of cells able to attach by preparing a standard curve. However, deviations within the

experiment were a cause for concern. I attempted to address this problem in many different ways. In addition to trying different cell numbers, I also tried different ways of preparing the bone slices. Initially our lab attempted to use the same area of each bone chip to culture the cells by drawing a circle by tracing with a wax pencil to contain the media. Later, I attempted to prepare bone slices of identical sizes by using a lathe to file a piece of bone into a cylinder and cutting slices from the cylindrical bone. I also attempted to use a hole-puncher in order to obtain slices that were the same size, but found that often times this would just fracture the bone. Finally, I cut slices of cortical bone that were of similar size and then used Adobe photoshop to calculate the exact area of each bone slice. I could then normalize the data by finding the number of cells per squared millimeter of bone. This method eliminated the need to create bone slices that were identical in size, which was functionally impossible due to the composition of the bone. Additionally, the assays were initially performed by lysing the cells on the bone and then moving aliquots of the lysate to a new plate in order to determine the luciferase activity. However, the results were not consistent using this method, regardless of cell number plated or length of incubation. To circumvent this issue, I collaborated with Dr. Kurt Zinn's lab in order to use their luminometer. This provided me the ability to lyse the cells directly on the bone slices and be able to determine luciferase activity without having to aliquot the lysate. However, these assays revealed that the stable knockdown of CD68 did not affect the ability of the cells lines to adhere to the bone (**Figure 6),** suggesting that CD68 either does not play a role in adhesion or that is among a group of proteins that have redundant function.

To further explore CD68's role in cell adhesion to bone, I forced expression of the two human CD68 isoforms, CD68.1 and CD68.2, in MCF-7 and MDA-MB-468, the breast cancer cell lines with very low endogenous expression of CD68. The functional differences between the two isoforms are currently unknown. Full-length human CD68 cDNAs were cloned into the pMX-puro vector and transiently transfected into 293GPG cells to produce virus. MCF-7 and MDA-MB-468 were then infected with the CD68 virus and selected for stable expression using puromycin. **Figure 7a** shows a Western Blot confirming CD68 expression at the protein level of both isoforms in both breast cancer cell lines. Cell adhesion to bone assays were performed and found that nothing more than background levels of luciferase activity occurred (as shown in **Figure 7b and c**), suggesting that forced expression of CD68 was not sufficient to allow MCF-7 and MDA-MB-468 bone adhesion.

b. Quantify and compare CD68 expression levels in normal mammary epithelial cells, breast cancer tissues from primary sites and breast cancer tissues from bone metastasis (Months 2-24).

I have applied for and obtained the Institutional review board (IRB) approval to proceed with this portion of the research. I am currently awaiting the normal and diseased tissues from our official collaborator Dr. Hue Luu in the Department of Surgery at the University of Chicago. Upon obtaining the tissues, CD68 expression will be examined by Immunohistochemistry. Preliminary studies from bone metastasis aspirates were negative for CD68, however this may be due to selection bias from the tissue harvesting. Slices of tumor bordering bone will provide a better way of imaging CD68 in breast cancer bone metastases.

c. Identify specific regions in the CD68 extracellular domain that are required for binding to bone to facilitate the elucidation of the molecular basis of the breast cancer -bone interaction and, more importantly, help develop more potent and specific monoclonal blocking antibodies that may be used as therapeutics for preventing and treating breast cancer bone metastasis (Months 8-18).

Plans of developing deletion mutants in order to determine the specific regions of bone interaction have been suspended due to the lack of association I found in Specific Aim 1 part A between CD68 and bone adhesion.

Task 2: Investigate whether CD68 is involved in breast cancer bone metastasis in vivo using animal models (Specific Aim 2) Months 12-34.

UAB's Institutional Animal Care and Use Committee (IACUC) have approved the proposed animal studies for the second and third year of this research in the second Specific Aim. These studies were delayed pending confirmation of CD68's role using *in vitro* cancer assays. However, I performed a small pilot study using the CD68-stable knockdown cells I generated and found that 4 weeks after intracardiac injection that only 33% injected with CD68-knockdown cell line developed metastatic lesions at an average of 0.67 metastatic lesions per mouse compared with 75-100% of mice injected with parental breast cancer cells or shRNA-negative control with an average of 3-3.2 metastatic lesions per mouse (**Figure 8)**. This was a pilot study so the limited number of mice do not provide enough to perform statistical analysis, however, this promising data highlights the need of a further understanding of CD68 in breast cancer bone metastasis.

Additional research presented in January 2012 report to redirect aims associated with the project:

Due to the lack of association I discovered between expression of CD68 and bone adhesion, I further investigated potential roles of CD68 that would explain the correlation between high CD68 levels and cancer recurrence and/or bone metastatic ability. It is also important to recall that CD68 has already been shown to be of clinical importance in breast cancer as it is among the 16 different genes on the Oncotype DX profile to predict risk of breast cancer recurrence (1). Increased expression of CD68, although the function remains unknown, in breast cancer tumors is correlated with an increased rate of recurrence, with CD68 having a hazard ratio of 1.11, which is the sixth highest estimate of relative risk among the 16 genes analyzed in Oncotype DX (1;2). This further supports my hypothesis that CD68 plays a role in the metastasis of breast cancer to bone.

For breast cancer bone metastasis to occur, it is essential that breast cancer cells have enhanced and/or long-term viability in the bone microenvironment. Autophagy has been proposed to be a pro-survival factor in cancer during starvation periods and/or hypoxic periods in order to delay apoptosis or non-apoptotic programmed cell death, such as autophagic cell death or lysosomal cell death. Autophagy is a "self-eating" process a cell can undergo in order to enhance viability during nutrient deprivation and is especially activated during amino acid starvation (3). Some cancer cells can undergo autophagy until they are just 1/3 of their original size but can resume proliferation within 24 hours if nutrients are restored (4). The autophagic pathway is a highly conserved pathway mediated by a double-membraned sac termed the autophagosome, an organelle formed by a small vesicular sac enclosing portions of the cell's cytoplasm. The autophagosome fuses with a lysosome to form an autolysosome, which degrades the materials within to be recycled in the cytoplasm or used for energy production (5). Therefore, lysosomes are an essential component of the autophagic process and could be implicated in long-term cell viability. Importantly, LAMP1 and LAMP 2, of which CD68 belongs to the same family, are involved in autophagy by regulating fusion of the lysosome to the autophagosome. LAMP regulation of autophagy is accomplished in at least three ways: redistributing the lysosomes within the cell, altering motility by cholesterol accumulation, and modifying Rab7 association with phagosomes, which is a protein necessary for phagolysosome fusion (6;7). Overexpression of LAMPs has been shown to protect cells from autophagic cell death, whereas decreased levels of LAMP1 and LAMP2 increase susceptibility to lysosomal cell death (8,9). Another LAMP family member, LAPTM4B, was recently

shown to play a pro-survival role in breast cancer by increasing resistance to anthracycline chemotherapies, by reducing drug entry into the nucleus (10). Therefore, members of the LAMP family may be important in supporting metastasis by enhancing long-term survival in breast cancer. Additionally, highly malignant tumor cells are found to have an increased cell surface expression of LAMP family members (9). I propose that because CD68 is a LAMP family member, it is involved in regulating autophagy to enhance viability in nutrient deprived conditions and/or lysosomal trafficking of drugs within the cell.

First, I addressed this by examining CD68's role in cell viability by stressing the cells by culturing them in PBS rather than media and controlling expression of CD68 by siRNA or forced expression. ATPlite assay was used to quantify the number of cells surviving after 1 hour or 4 hours in PBS. There was no statistical difference between the CD68 knockdown cells and the control cells at either timepoint (**Figure 9**), although there appeared to be a difference in cell morphology. To further examine molecular differences I wanted to investigate CD68's potential role in regulating autophagy, as it is a lysosomal associated process that has been previously linked to cancer cell survival. To this end, autophagic pathways were examined by LC3 immunoblotting and quantification of acidic vesicular organelles with acridine orange staining (**Figure 10).** However, no difference was seen in either timepoint when comparing the knockdown cells to scramble control. Furthermore, there were no notable morphologic changes by serum starvation, including cytoplasmic and nuclear shrinkage, chromatin condensation, cytoplasmic blebbing with maintenance of the integrity of cell membrane, to determine autophagy.

Lastly, I examined if CD68 acts as a chemoresistant factor by adding the chemotherapeutic drug Doxorubicin to the cultures 24 hours after seeding the cell lines. Cells were exposed to a series of concentrations of the drug for an additional 48 hours. The percentage of viable cells in drug-treated wells as compared to media-treated control wells were plated as a drug-dose dependent survival curve and the IC_{50} (dose causing 50% reduction of viable cells) was determined to compare the scramble control to the CD68-knockdown. There was some variation in these studies, highlighting the fact that passage number, and condition of the cells at plating is vitally important. Some assays would result in a significant decrease in the number of cells surviving when CD68 was knocked down while other assays would show no difference. **Figure 11** shows a representative graph suggesting there is a trend and that more studies are needed to determine if CD68 could have this important potential role as a chemoresistant molecule.

Due to the uncertainty of finding a publishable function of CD68 in a timely manner to allow me to obtain my PhD, I also began to look at the role of a breast cancer secreted cytokine called IL-11 and its function on osteoclasts and breast cancer bone metastasis. Higher rates of bone metastasis occur in human breast cancer tumors expressing IL-11 (11), but the specific mechanisms of action on osteoclasts are controversial (12-16). I determined that breast cancer secreted IL-11 was unable to directly support osteoclast survival or formation in sub-optimal RANKL conditions but instead supports or maintains a population of osteoclast progenitors. These findings clarify IL-11's specific role in breast cancer bone metastasis, and future studies are warranted to address the therapeutic potential of IL-11 for treating and preventing breast cancer induced osteolysis. My recently published paper concerning things findings is attached on page 33 of the appendix.

Key research accomplishments

- CD68 expression levels in MDA-MB-231, MDA-MB-435, MDA-MB-468, and MCF-7 breast cancer cell lines positively correlate with cells' ability to adhere to bone slices

- Stable knockdown of CD68 was achieved using shRNA
- Adhesion to bone was not affected when expression of CD68 was inhibited using shRNA in either MDA-MB-231 or MDA-MB-435.
- Forced expression of high levels of CD68 was achieved in MDA-MB-468 and MCF-7, lines that intrinsically express very low levels of CD68, but was not sufficient for adhesion.
- Different techniques of bone adhesion assays were performed to overcome experimental difficulties
- Preliminary animal model studies suggest that the knockdown of CD68 renders breast cancer cell lines less metastatic
- Knockdown of CD68 does not appear to have an overt action when breast cancer is stressed by culturing in PBS conditions
- My preliminary work suggests the potential for CD68 acting as a chemoresistance molecule for breast cancer
- Overall, my work suggests that because CD68 can be expressed directly by breast cancer, it should be reevaluated as a macrophage marker, particularly when looking at tumor histology.
- Breast cancer secreted IL-11 plays an important role in osteoclastogenesis by stimulating the development and/or survival of osteoclast progenitor cells
- These findings have not only provided a better understanding of the role of IL-11 in breast cancer bone metastasis but also laid a foundation for future investigations to address therapeutic targeting of IL-11 for treating and preventing breast cancer induced osteolysis.

Reportable outcomes

Publications related to aims:

- "Characterizing the roles of IL-11 and CD68 in breast cancer bone metastasis" Erin M McCoy. A dissertation, accepted for publication by ProQuest/UMI on January 2nd, 2013.

Publications not related to aims:

- **McCoy EM,** Hong H, Pruitt HC, Feng X. IL-11 produced by breast cancer cells augments osteoclastogenesis by sustaining the pool of osteoclast progenitor cells. BMC Cancer. 2013 **13**:16.
- Ashley JW**, McCoy EM**, Clements DA, Shi Z, Chen T, Feng X. Development of cell-based high-throughput assays for the identification of inhibitors of receptor activator of nuclear factor-kappa B signaling. *Assay Drug Dev Technol* 9(1):40-49, 2011.

Abstracts related to aims selected for poster presentations

- "Potential Role of CD68 in breast cancer bone metastasis" **Erin M McCoy**. Zhenqi Shi, Xu Feng. *American Society for Bone and Mineral Research*. Poster presented in Toronto, Canada in the cancer section; October 15-19, 2010.
- "Regulation of Breast Cancer Attachment to Bone by CD68**" Erin M. McCoy**, Jason W Ashley, Zhenqi Shi, Xu Feng. *Dept of Pathology, University of Alabama at Birmingham.* Poster presented at Pathology Graduate Student Research Day, UAB; October 2010**.**

Awards:

- Pathology graduate student travel grant awarded for the presentation at American Society for Bone and Mineral Research; October 15-19, 2010.
- First place - poster presentations for Pathology Graduate Student Research Day, October 2010. "Potential Role of CD68 in breast cancer bone metastasis." Erin M. McCoy. *Dept of Pathology, University of Alabama at Birmingham.*

Other:

- Presented "Breast cancer secreted IL-11 augments osteoclastogenesis by sustaining the pool of osteoclast progenitor cells" poster at American Society for Bone and Mineral Research, Minneapolis, MN; October, 2012
- Attended Annual meeting of American Association for Cancer Research, Florida, USA; April 2011.
- Successfully passed my doctoral defense examination by my thesis committee and gained approval of my dissertation "Characterizing the roles of IL-11 and CD68 in breast cancer bone metastasis" in December 2012.

Conclusions

Through the course of this research so far, I have been able to determine that CD68 is upregulated in certain breast cancer cells lines (MDA-MB-231 and MDA-MB-435) that have a higher capacity adhere to bone slices *in vitro* and to metastasize to bone *in vivo*. Paired with the clinical use of CD68 as a recurrence risk factor for breast cancer, the need to find the specific role(s) for CD68 is critical in order to better understand and treat breast cancer metastasis. Although initially we found CD68 through a phage display bone adhesion assay and there is a correlation between expression of CD68 and bone adhesion abilities of breast cancer cells lines, CD68 does not appear to be the critical adhesion molecule that allows breast cancer to attach to bone. Therefore, I began a thorough literature review to investigate other potential roles that CD68 may play in breast cancer metastasis and determined that, because CD68 is a LAMP, there is great potential for it to be involved in the pro-metastatic autophagy and/or chemotherapeutic resistance through autophagy. The important finding of breast cancer expression of CD68 suggests that CD68 should be reevaluated as a macrophage marker, particularly when looking at tumor histology. This work emphasizes the importance of investigating CD68's direct role in breast cancer, which strengthens CD68's high relative risk correlation on the Oncotype DX breast cancer screening assay and could potentially lead to opportunities for advances in therapeutics.

Secondly, I've shown that breast cancer secreted Il-11 targets osteoclast progenitor cells by promoting and/or contributing o the survival of osteoclast progenitor cells, which could contribute to the vicious cycle of bone metastasis. This leads to potential further investigation to determine IL-11's role as a therapeutic target. Another point for consideration is that recombinant IL-11 is approved by the US Food and Drug Administration to treat patients suffering from severe thrombocytopenia during high-dose chemotherapy, due to its hematopoietic effects of stimulating megakaryocyte production and ability to reduce mucositis, and therefore should be examined to ensure side effects don't include bone metastases (17).

Because the bone is the most common site of distant metastases in breast cancer, and the bone metastases account for serious morbidities and a poor prognosis, it is crucial to elucidate the precise molecular mechanisms in order to develop more specific therapeutics and help more patients. The work I've accomplished provides novel insights into the mechanisms by which breast cancer may increase bone destruction, specifically IL-11 maintaining osteoclast progenitors, and the expression of CD68 by breast cancer which may influence its use as a macrophage marker and open doorways into its biological relevance within the cancer tumors. Both of these molecules need to be further investigated for their potential therapeutic value. Though there is much more work to be done, the work on this grant contributes to the strong base of work dissecting the relationship between breast cancer bone metastasis and the body's bone resorbing cell, the osteoclast.

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Appendices

Personnel receiving pay from the research effort: Erin McCoy

Accepted abstract for American Society for Bone and Mineral Research 2012

Poster presented in Minneapolis, MN in the cancer section; October 4-7, 2012.

Interleukin (IL)-11 Promotes Osteoclastogenesis by Stimulating Differentiation and Survival of Osteoclast Progenitor Cells

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Bone metastases occur in more than 80% of patients with advanced breast cancers and are responsible for the worst morbidities of the disease. The cytokine interleukin (IL)-11, which is known to be produced by breast cancer, has been shown to promote osteoclast formation, allowing for excessive bone resorption. The actions of IL-11 in the osteoclast formation process have not been fully elucidated, with some groups suggesting a receptor activator of NF-κB ligand (RANKL) – independent role in osteoclast formation while others show that osteoclast formation is enhanced by IL-11 only when osteoblasts are present in the culture. We sought to further investigate the role of IL-11 in supporting osteoclast formation, function and survival. To this end, we first determined whether IL-11 was sufficient to support RANKL-independent osteoclast formation using murine bone marrow macrophages and found that IL-11 as high as 100ng/ml was unable to induce osteoclast formation. Given that several other cytokines such as IL-1 and tumor necrosis factor α (TNF-α) can promote osteoclastogenesis in the presence of permissive levels of RANKL, we next investigated whether IL-11 can do so, too. Our data indicate that, unlike IL-1 and TNF-α, IL-11 failed to enhance osteoclast formation in the presence of permissive levels of RANKL. We then pretreated bone marrow macrophages with either macrophage-colony stimulating factor (M-CSF), PBS, or IL-11 for 4 days prior to treatment with RANKL and MCSF. We found that while freshly isolated murine bone marrow cells treated with PBS only died by day 5, IL-11 was capable of supporting survival of a small population of bone marrow cells. Importantly, the IL-11-dependent cells formed osteoclasts upon treatment with RANKL and MCSF. These data suggest that IL-11 plays an important role in osteoclastogenesis by stimulating survival and/or differentiation of osteoclast progenitor cells. These studies have revealed a new mechanism by which IL-11 exerts its impact on osteoclast formation/function. Secondarily, these findings have laid a foundation for future investigations to clarify IL-11's specific role in breast cancer bone metastasis, which may provide novel insights into the development of breast cancer bone metastasis but also address therapeutic potential of IL-11 for treating and preventing breast cancer induced osteolysis.

Accepted abstract for American Society for Bone and Mineral Research 2010

"Potential Role of CD68 in breast cancer bone metastasis" Erin M McCoy. Zhenqi Shi, Xu Feng. *American Society for Bone and Mineral Research.* Poster presented in Toronto, Canada in the cancer section; October 15-19, 2010.

Bone is a common site of metastasis in breast cancer patients, leading to serious clinical consequences and a poor prognosis. The molecular mechanisms regulating this preferential metastasis of breast cancer to bone have not yet been fully elucidated. Using a Phage Display-based approach by biopanning with bone slices, our lab recently identified macrosialin as a key mediator of osteoclast attachment to bone. Interestingly, we also found that CD68, the human homologue of macrosialin, is highly expressed in certain breast cancer cell lines. We hypothesize that CD68 mediates attachment of breast cancer cells onto bone matrix, subsequently regulating bone metastasis. Here we demonstrate that expression of CD68 is significantly increased in breast cancer cell lines that exhibit bone metastasis capacity, as compared to breast cancer cell lines that do not metastasize to bone. Furthermore, anti-CD68 antibody significantly blocks the attachment of breast cancer cells to bone *in vitro*, supporting that CD68 plays a key role in breast cancer cell interaction with bone. These data indicate that CD68 plays a critical role in mediating breast cancer cell attachment to bone. We are currently characterizing this interaction by using shRNA to knock down CD68 expression in breast cancer cells and determining bone metastatic capability *in vitro*, as well as *in vivo*. Additionally we are also expressing CD68 in breast cancer cell lines that do not endogenously express it, in order to determine if CD68 will render them capable of attachment to bone. These ongoing studies may further provide direct and convincing data.

CURRICULUM VITAE

Erin Mills McCoy, B.S.

DEMOGRAPHIC INFORMATION

Current Position

Howard Hughes Med-to-Grad Pre-doctoral fellow – Department of Pathology, Division of Cellular and Molecular Pathology, University of Alabama at Birmingham

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Place of Birth:	Hattiesburg, MS
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Education:

6/07 - present	Howard Hughes Med-to-Grad Pre-doctoral fellow Department of Pathology Division of Cellular and Molecular Pathology University of Alabama at Birmingham Overall GPA – 4.0, 213 hours completed
5/03 – 5/07	B.S. - Biological Sciences B.S. – Microbiology Minor – Biochemistry Mississippi State University; Starkville, MS Overall GPA: 3.97, 143 hours completed
08/06	GRE: Verbal: 530; Quantitative: 740; Total 1270

Honors and Activities:

Graduate:

Department of Defense Pre-doctoral Breast Cancer Fellowship award: 2011- 2014.

Pathology graduate student travel grant awarded for the presentation at American Society for Bone and Mineral Research; October 15-19, 2010.

Carmichael Scholarship for Academic Excellence: 2009-2011; competitively renewed for 2010-2011: Awarded

Most Outstanding Graduate Student through Service Award: 2008-2009 as chosen by a vote from all peers in the Pathology Graduate Student Department

First place – poster presentation for Pathology Graduate Student Research Day, October 2010**. "Regulation of Breast Cancer Attachment to Bone by CD68"** Erin M. McCoy, Jason W Ashley, Zhenqi Shi, Xu Feng. *Dept of Pathology, University of Alabama at Birmingham.*

Second place - oral presentations for Pathology Graduate Student Research Day, October 2008. "Characterizing the role of CD68 in mediation of breast cancer attachment to bone." Erin M. McCoy. *Dept of Pathology, University of Alabama at Birmingham.*

Howard Hughes Med-to-Grad Fellowship 2007-present: Ph.D. Training fellowship for Translational Research and Drug Discovery at University of Alabama at Birmingham. This fellowship is awarded to the top incoming students in the biomedical sciences graduate programs and supports the students for the first 16-months of their graduate career.

Undergraduate:

President's list (every semester) - Mississippi State University
Phi Beta Kappa, Society of Scholars, inducted 2006
Phi Kappa Phi, inducted 2006
Mortar Board, Treasurer 2006 - 2007
Mabry-Clark Memorial Scholar, selected as one of two students from MSU, 2006
Sigma Phi Lambda service sorority, Vice President 2006
Fred and Mary Koch Scholarship, 2004-present
National Merit Georgia Pacific Scholarship, 2003-2007
Robert C. Byrd Scholarship, 2003-2007
Mississippi Eminent Scholars Grant, 2003-2007
MSU Academic Scholarship, 2003-2007

RESEARCH ACTIVITIES

Major Research Interests:

July 2008 – present. We are interested in understanding the molecular and cellular mechanisms of breast cancer metastasis to bone; specifically adhesion molecules that allow interactions between breast cancer cells and bone surface and viability factors allowing for survival in the bone microenvironment.

Past Research Experience:

Research Assistant: Mississippi Genomic Exploration Laboratory, Department of Plant and Soil Sciences, Mississippi State University, August 2006 – March 2007: Participated in research to complete a bacterial artificial chromosome library for the genomic sequencing of the loblolly pine tree species.

Research Assistant: Department of Biological Sciences, Mississippi State University, August 2006 – Jan 2007: Participated in FIV research to monitor acute serological and virological changes in the peripheral circulation of FIV-infected cats.

Program for Research Experience in Pathology, University of Alabama at Birmingham, May 2006 – July 2006: Successfully constructed a luciferase reporter to be further used in a cell-based high throughput screening for anti-resorptive compounds.

Research Assistant: Department of Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, May 2005 – May 2006: Participated in research on the risk factors of *Salmonella* in poultry farms and processing plants.

EDUCATIONAL ACTIVITIES

Publications:

IL-11 produced by breast cancer cells augments osteoclastogenesis by sustaining the pool of osteoclast progenitor cells. McCoy EM, Hong H, Pruitt HC, Feng X. BMC Cancer. 2013, 13:16. Doi: 10.1185/1471-2407-13-16

Development of cell-based high-throughput assays for the identification of inhibitors of receptor activator of nuclear factor-kappa B signaling. Ashley JW**, McCoy EM**, Clements DA, Shi Z, Chen T, Feng X. Assay Drug Dev Technol. 2011 Feb;9(1):40-9.

Accepted Abstracts/Presentations:

"IL-11 promotes osteoclastogenesis by stimulating differentiation and/or survival of osteoclast progenitor cells. **Erin M McCoy,** Huixian Hong, Xu Feng. *American Society for Bone and Mineral Research.* Poster presented in Minneapolis, Minnesota in the cancer section; October 12-15, 2012.

"Potential Role of CD68 in breast cancer bone metastasis" **Erin M McCoy**. Zhenqi Shi, Xu Feng. *American Society for Bone and Mineral Research*. Poster presented in Toronto, Canada in the cancer section; October 15-19, 2010.

"Potential Role of CD68 in breast cancer bone metastasis." **Erin M McCoy**. Zhenqi Shi, Xu Feng. Poster presented for Pathology Graduate Student Research Day, October 2010. *Dept of Pathology, University of Alabama at Birmingham*. (1st place poster presentation)

"Regulation of Breast Cancer Attachment to Bone by CD68" **Erin M. McCoy,** Zhenqi Shi, Xu Feng. *Department of Pathology, University of Alabama at Birmingham.* Poster presented at UAB Comprehensive Cancer Retreat, Birmingham, AL; November 2008.

"Characterizing the role of CD68 in mediation of breast cancer attachment to bone" **Erin M. McCoy**, Zhenqi Shi, Xu Feng. Oral presentation (2nd place) – Pathology Graduate Student Research Day, October 2008.

"Cell-Based Assays for Identifying Inhibitors of Bone Loss" JW Ashley, Z Shi, **EM McCoy**, DA Clements, X Feng. *Department of Pathology, University of Alabama at Birmingham.* Poster presented at the Regional Peer Cluster Conference for Howard Hughes Med-to-Grad Fellows, Birmingham, AL; August 2008.

"Disruption of VPS34 expression *in vivo* reveals its novel role in embryonic development." **Erin M. McCoy** and Jianhua Zhang. *Department of Pathology, University of Alabama at Birmingham.* Oral presentation open to all departments, May 2008.

"Deletions of alpha-Calmodulin Kinase II in Osteoblasts Reveals Its Novel Role in Bone Remodeling" **Erin M. McCoy** and Majd Zayzafoon. *Department of Pathology, University of Alabama at Birmingham.* Oral presentation open to all departments, March 2008.

"Identification of c-Src Motifs Essential for Osteoclast Function." **Erin M. McCoy** and Xu Feng. *Department of Pathology, University of Alabama at Birmingham.* Oral presentation open to all departments, December 2007.

"Analysis of Recombinant ssM13mp18 RF pGFPuv Using DNA sequencing and Detection of pGFPuv and pUC by Southern Blotting. **Erin E. Mills.** Oral presentation – Department of Biochemistry, Mississippi State University, April 2007.

"Kinetic Studies of Chicken Muscle Lactate Dehydrogenase: Inhibition using Oxalate, Irreversible Inhibition using Butanedione, and pH Characterization. **Erin E. Mills.** Oral presentation – Department of Biochemistry, Mississippi State University, Dec 2006.

"Virological and Serological Consequences of Acute Feline immunodeficiency Virus (FIV) Infection" Crystal Boudreaux, **Erin Mills,** Matt Bramuchi, Veronica Scott, Nikki Lockett, Brittany Clay, and Karen S. Coats. *Department of Biological Sciences, Mississippi State University.* Poster presented at the South Central Branch of the American Society of Microbiology, Baton Rouge, LA; November 3-4, 2006.

"Construction of a Luciferase Reporter for Establishing a Cell-based High Throughput Screening Assay System to Identify New Antiresorptive Drugs" **Erin E. Mills**, Zhenqi Shi, and Xu Feng.

Department of Pathology, University of Alabama at Birmingham. Poster presented at the closing ceremony for the *Program for Research Experience in Pathology, UAB,* July 2006.

Classroom Instruction:

English as a Second Language Teacher Training: 14 hour teacher training class, received certification January 30, 2010. To date: 24 hours in classroom.

Instructor: General Chemisty, CH121-03, Birmingham Southern College Department of Chemistry, Fall 2008.

Teaching Assistant: Practices in the Physiology of Reproduction, PHY 4611, Mississippi State University-College of Veterinary Medicine, Fall 2005.

Tutoring Positions:

Summer 2010
Summer peer mentor, McNair Scholars Research Program, UAB, Birmingham, AL

Fall 2009 – Spring 2011
Fundamentals of Biochemistry; McNair Scholars Program; UAB, Birmingham, AL

Fall 2006 – Summer 2007
Plant and Animal Biology. Mississippi State University; Starkville, MS

Summer 2004
Chemistry I & II, Organic Chemistry I. Jones County Junior College; Ellisville, MS

Judging Activities:

UAB Center for Community Outreach Development (CORD) Summer Science Institute Closing Ceremonies Poster Competition, Birmingham, AL; August, 2009.

ORGANIZATION ACTIVITIES

Institutional Committees:

August 2008 – Fall 2009
Senator representative for Pathology in the Graduate Student Association; chair of Career Day committee, member of Student Health committee.

July 2006 – May 2007.
Member of the Institutional Animal Care and Use Committee – Student body representative for Mississippi State University.

Professional Societies:

American Association for Cancer Research, 2009-present
Metastasis Research Society, 2008-present
American Association for the Advancement of Science, 2007 - present

Committees/Offices held

Team Captain for the UAB Pathology Department – Komen Breast Cancer Race for the Cure, October 2008 and October 2009, Birmingham, AL.

Co-chair and organizer for the First Annual Howard Hughes Medical to Graduate Fellow Peer Cluster Conference (UAB, Baylor, and Rice University), August 2008, Birmingham, AL .

Co-chair of Activities Committee for Howard Hughes Med-to-Grad Fellows. Activities include certification and opportunities for middle school and high school tutoring/mentoring programs to facilitate scientific interest at an early age.

Chair of Volunteer committee: Sigma Phi Lambda 2005-2007. Organized and assisted with events such as food and clothing drives, Hurricane Katrina outreach programs, and Habitat for Humanity programs.

OTHER EXPERIENCE:

Research Assistant: Atlanta Zoo, Atlanta, GA. December 2005. Assisted with a digestibility trial in pandas. Collected fecal matter and ortfs (rejected bamboo), sub-sampled and separated by plant species. Selected as one of two students from a pool of 135 students.

Dairy Farm Research Assistant: Dairy Research Center, Mississippi State University. May 2005 through December 2006. Assisted in the collection of milk samples for research and maintained the separation between different experimental groups within the herd.

Veterinary/Surgical Assistant: Southern Pines Animal Shelter, Hattiesburg, MS. May 2001 through December 2004: Collected ear, skin, and tissue cultures, performed stains, autoclaved surgical equipment. Gained familiarity with sterile and aseptic techniques.

SUPPORTING DATA

FIGURE 1

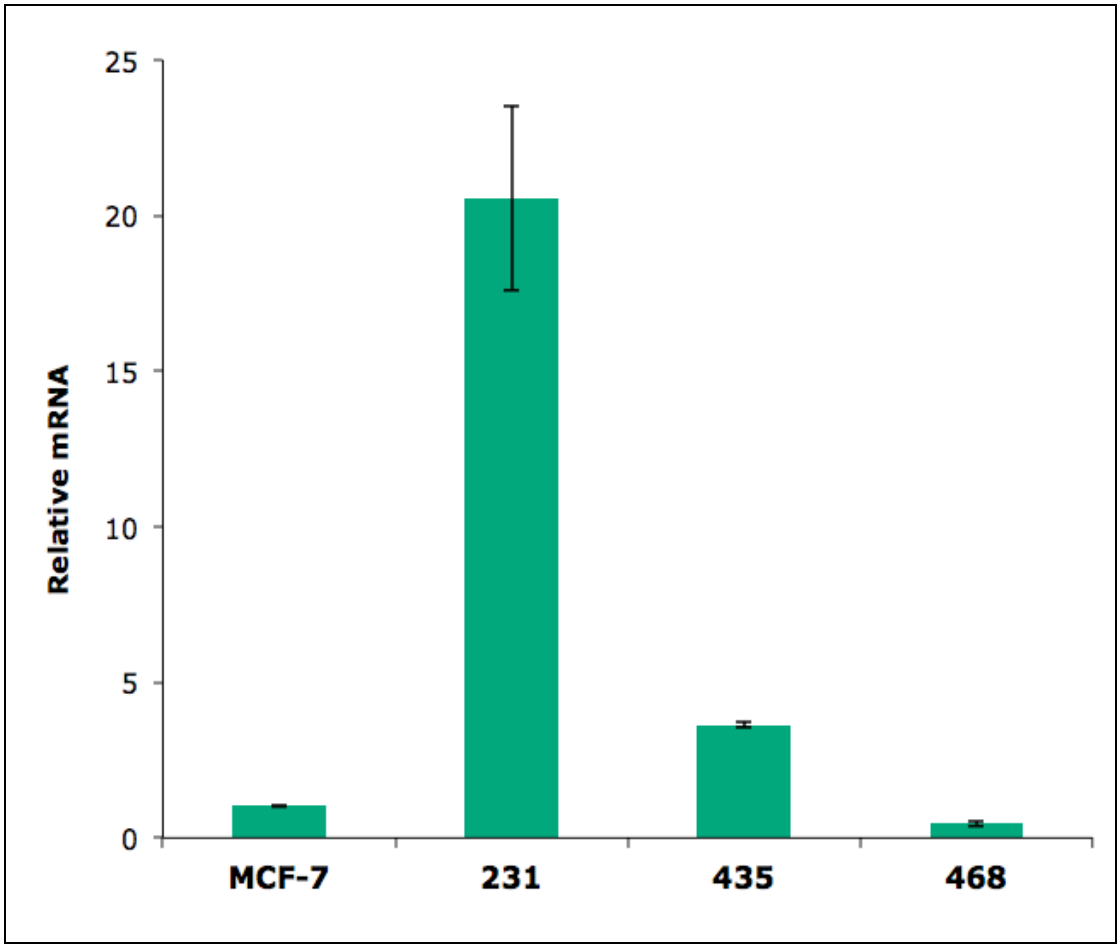

Figure 1: Expression of CD68 mRNA is upregulated in osteolytic breast cancer cell lines. mRNA transcripts of CD68 are found at levels relatively higher in MDA-MB-231 and MDA-MB-435, cell lines capable of osteolytic metastasis in *in vivo* metastasis studies, as compared to non-osteolytic cell lines, MDA-MB-468 and MCF-7. Total RNA was extracted, reverse transcribed and assessed using SYBR-green real-time RT-PCR in an Applied Biosystems 7500 Real-Time PCR System and normalized to S9 (n=3; repeated in triplicate).

FIGURE 2

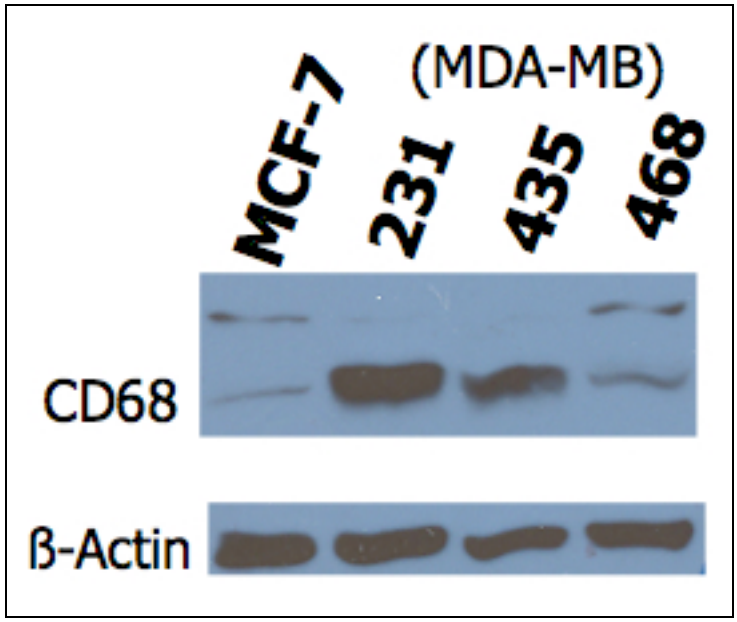

Figure 2: Expression of CD68 protein is upregulated in osteolytic breast cancer cell lines. Western blotting analysis of CD68 reveals that protein levels of CD68 are found at levels relatively higher in MDA-MB-231 and MDA-MB-435, cell lines capable of osteolytic metastasis in *in vivo* metastasis studies, as compared to non-osteolytic cell lines, MDA-MB-468 and MCF-7. Actin was used as a loading control. Experiment was repeated 9 times.

FIGURE 3

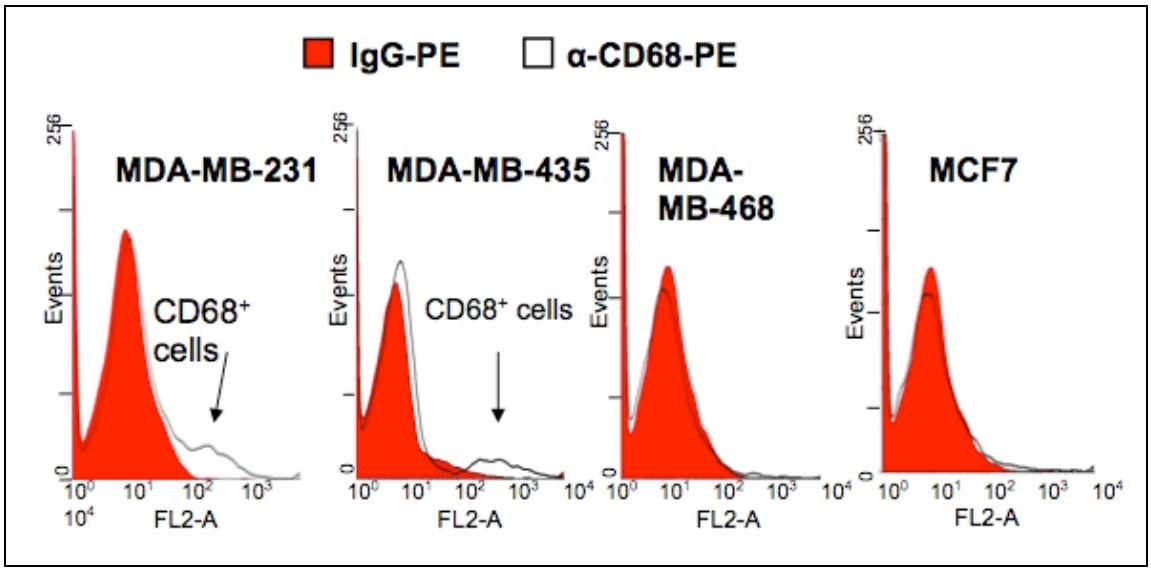

Figure 3: Surface expression of CD68 is limited on breast cancer cell lines.
Antibodies conjugated to phycoerthryin against CD68 and IgG were used to perform flow cytometry of the four breast cancer cell lines to detect surface expression of CD68. MDA-MB-231 and MDA-MB-435 had small populations of cells that expressed CD68 on the surface, but it is seen primarily to be an intercellular molecule in these breast cancer lines although cycling from intracellular to surface expression is possible. As expected, due to low overall expression, no surface expression as seen for either MDA-MB-468 or MCF7.

FIGURE 4

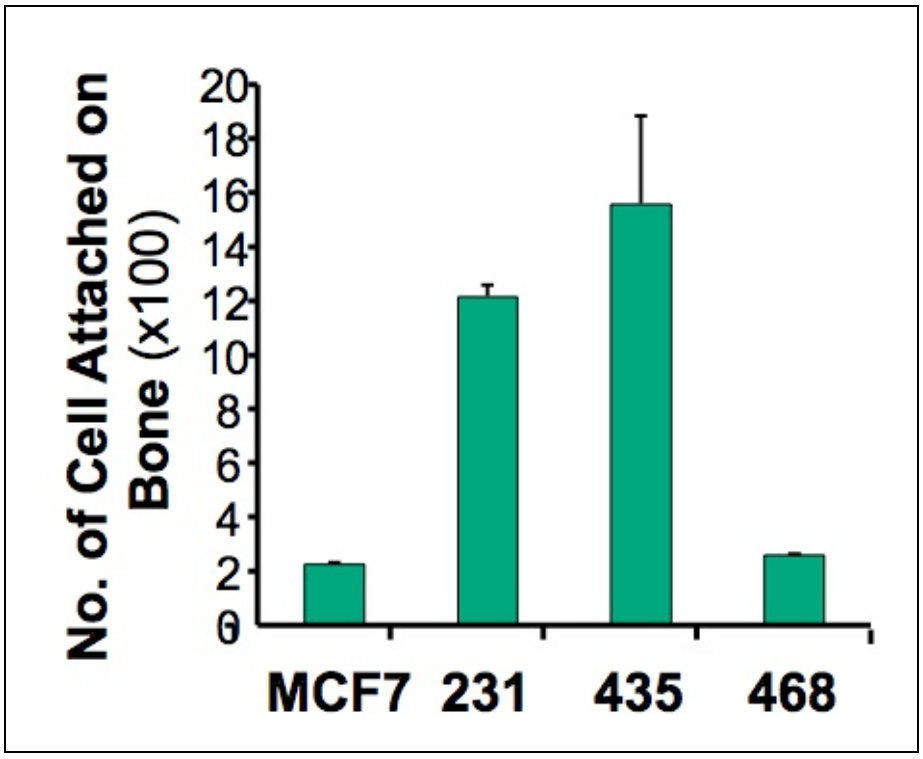

Figure 4: CD68 levels correlate with adhesion of breast cancer cells onto bone *in vitro*. Breast cancer cells lines MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-468 were cultured to 90% confluency and then lifted using 2mM EDTA. Cells were incubated on bone slices for one hour, and assay was performed as described in Materials and Methods. Osteolytic breast cancer cell lines, which have an increased expression of CD68 compared with MCF7 and MDA-MB-468, showed a higher ability to attach to the bone slices, suggesting that CD68 expression correlated with adhesion ability.

FIGURE 5

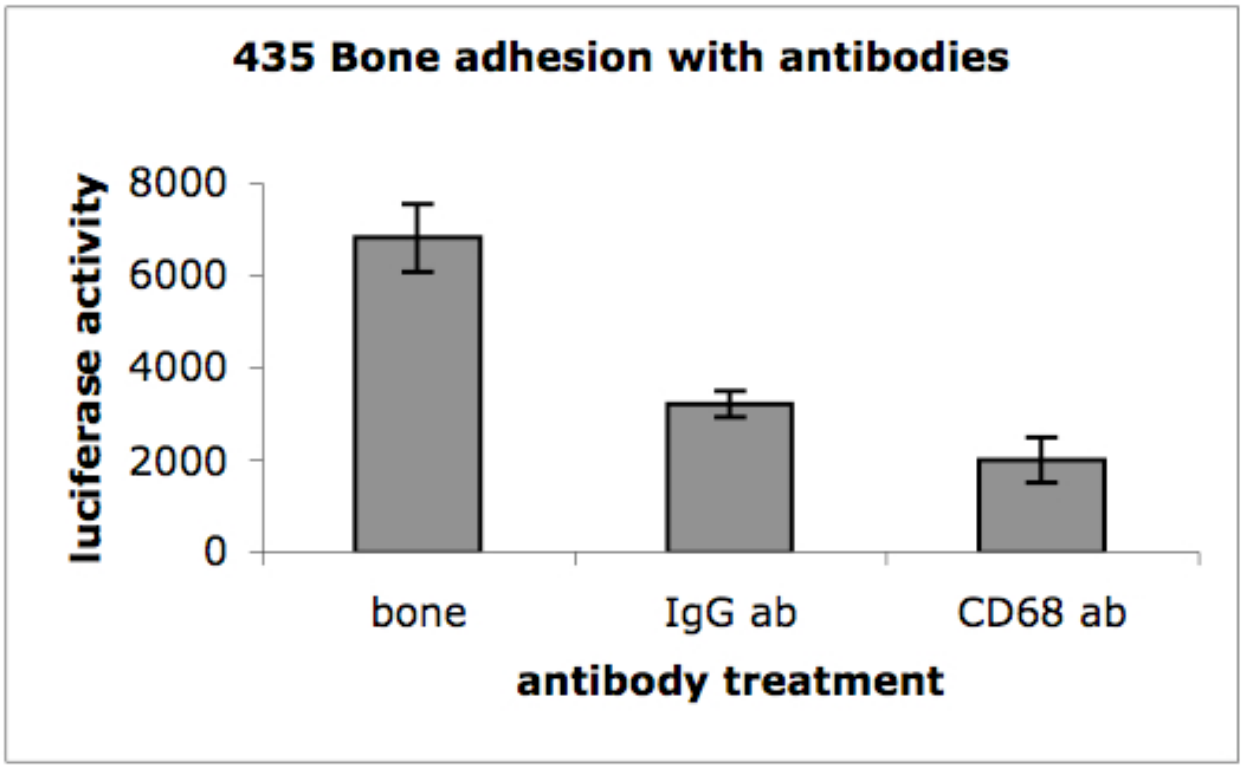

Figure 5: Bone adhesion assay showing decrease in adhesion with both the IgG control antibody as well as the CD68 antibody (Santa Cruz antibody sc-17832, mouse monoclonal human CD68)(E-11). Cells were treated for 30 minutes with 10μg of either antibody and then cultured on the bone slices for 1 hour. A decrease in adhesion was shown with the CD68 antibody, but confounded by the decrease in the IgG control antibody group.

FIGURE 6

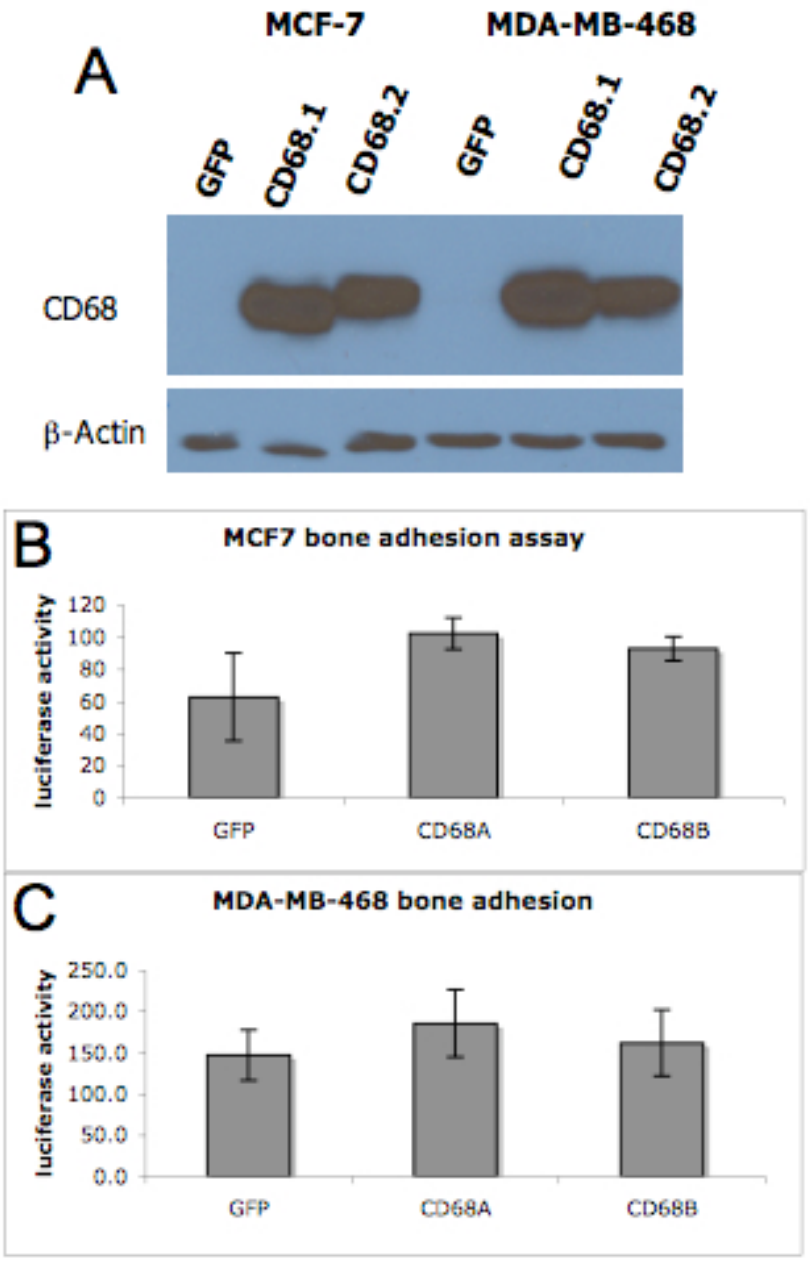

Figure 6: Forced expression of CD68 in MCF-7 and MDA-MB-468 is not sufficient for bone adhesion. Breast cancer cells lines MCF-7 and MDA-MB-468 were stably transfected with vectors containing two isoforms of CD68 (68.1 and 68.2). After confirming expression by Western Blotting (A), the cells were used to perform bone adhesion assays as described in detail in the Materials and Methods section. B, C) No significant difference in bone adhesion ability was found in either cell line when comparing the GFP transfected to the CD68 forced expression lines.

FIGURE 7

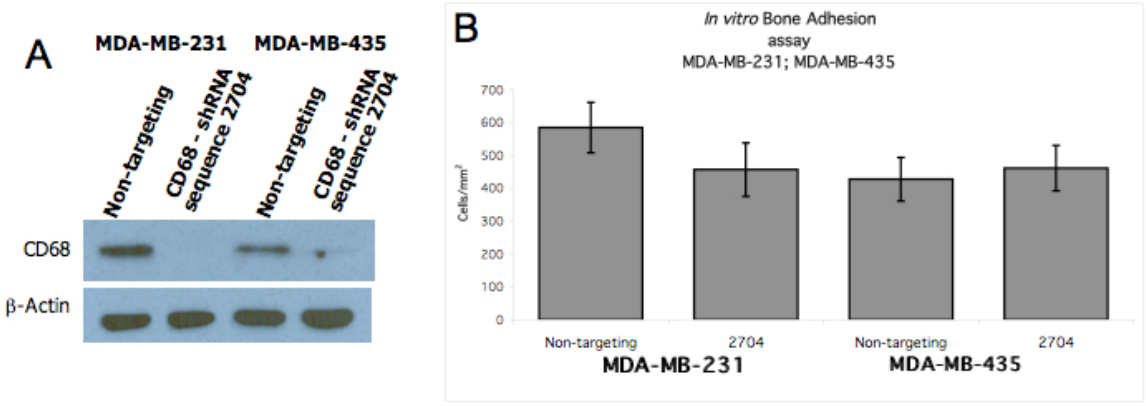

Figure 7. Bone adhesion assay using stable knockdown of CD68. A) Western blotting of CD68 protein (Santa Cruz antibody sc-17832, mouse monocloncal human CD68 (E-11) showing downregulation of CD68 by knockdown B) Bone adhesion assay comparing non-targeting to CD68-knockdown in MDA-MB-231 and MDA-MB-435. Error bars represent standard error, p-values were not significant (0.3 for MDA-MB-231 and 0.8 for MDA-MB-435)

FIGURE 8

Week 4	n	% with mets	Ave mets/ mouse
parental	4	75%	3
kd	3	33.3%	0.67
neg	5	100%	3.2

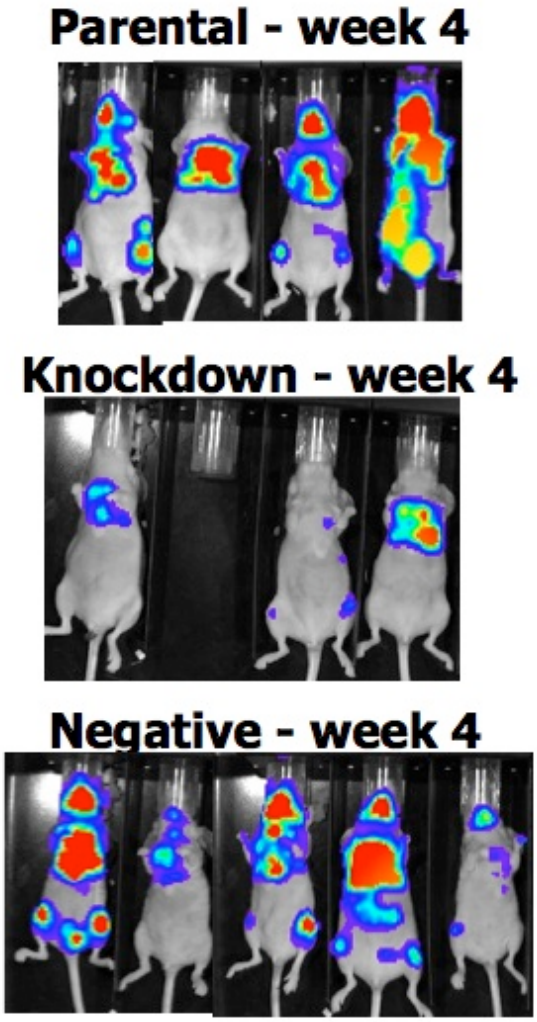

Figure 8. Pilot animal model study. CD68 knockdown cancer cell lines produced metastasis in only 1 of 3 animals compared with 5 of 5 for negative control or 3 of 4 for parental cell line. Furthermore, the average metastasic lesions per mouse was only 0.67 for the CD68 knockdown compared with 3 and 3.2 for parental and negative control, respectively. Statistical analysis could not be performed due to low number of animals but this encouraging data shows the necessity of determining CD68's role in breast cancer bone metastasis.

Figure 9

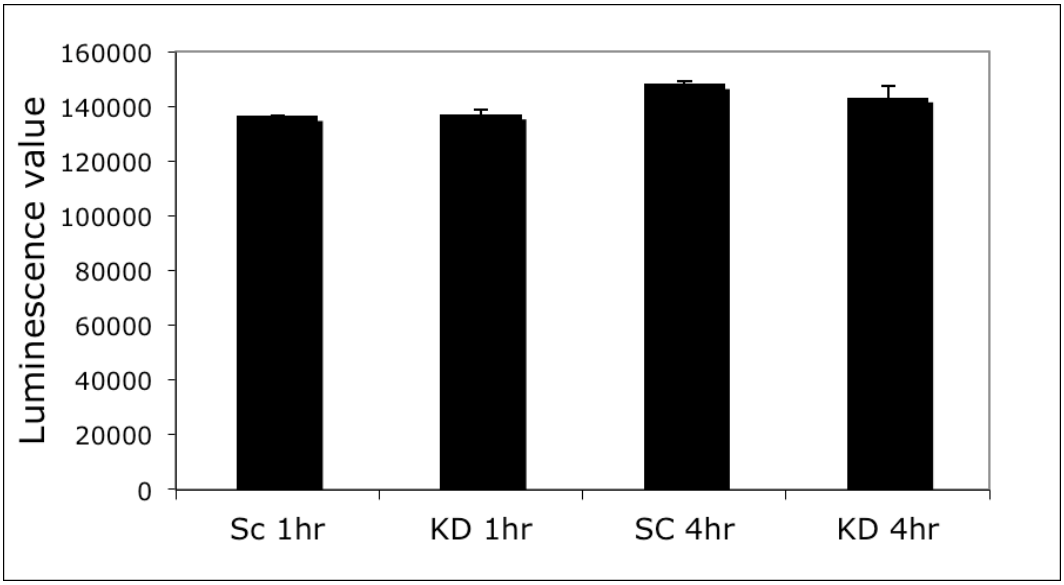

Figure 9: Viability of breast cancer cells when challenged by PBS culture. MDA-MB-231 breast cancer cell lines were stably transfected with shRNA against CD68 to knockdown expression (KD) or with scrambled shRNA as control (SC). Media was then washed from cells and replaced with PBS containing no added nutrients. Cells were maintained for 1 or 4 hours and then lysed using the ATPlite system to determine the viable cells. There was no difference seen at either timepoint.

FIGURE 10

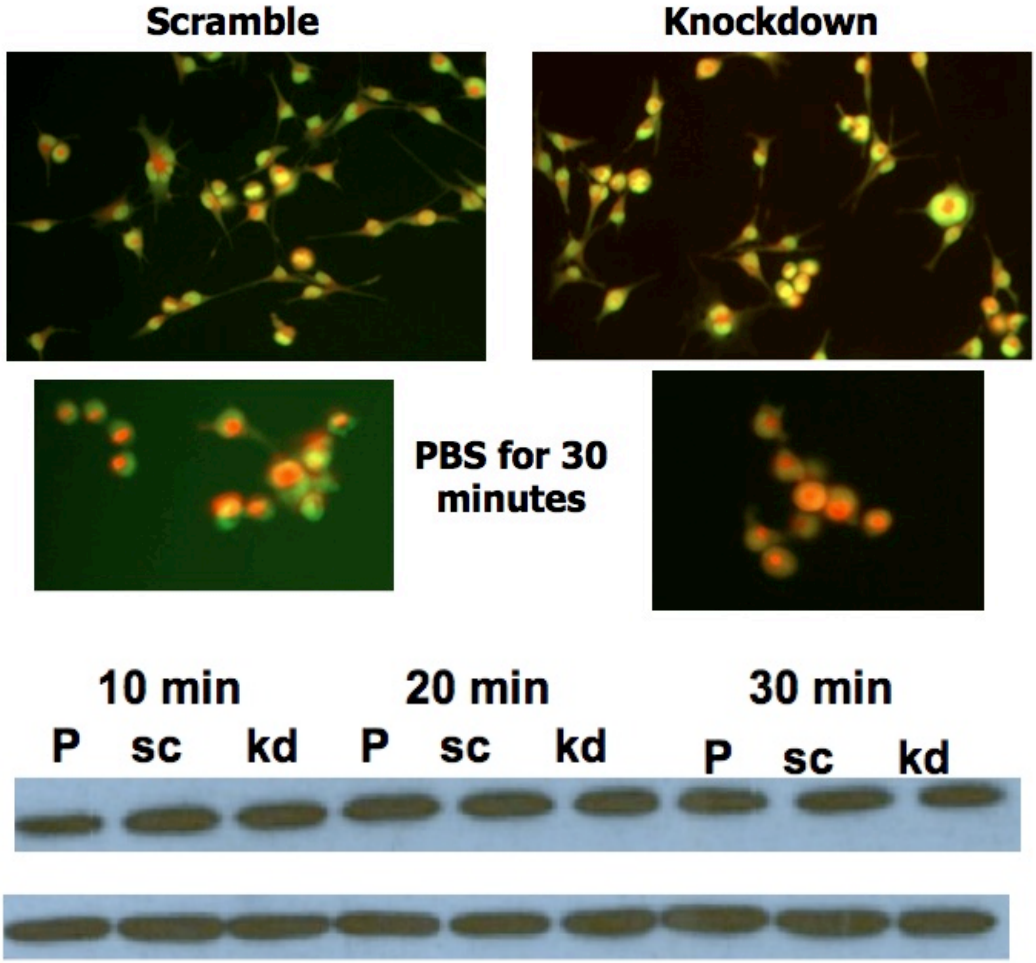

Figure 10: Acradine orange staining and LC3 immunoblotting for autophagy. No difference in acridine orange staining was seen at normal conditions or after culturing in PBS for 30 minutes when comparing breast cancer cell lines MDA-MB-231 with CD68 knockdown to MDA-MB-231 with Scramble control. Furthermore there was no difference in the amount of LC3 present in parental cell (P), scramble cells (sc), or knockdown cells (kd) at 10, 20, or 30 minutes of PBS. Taken together these data indicate no difference in autophagy under these conditions.

FIGURE 11

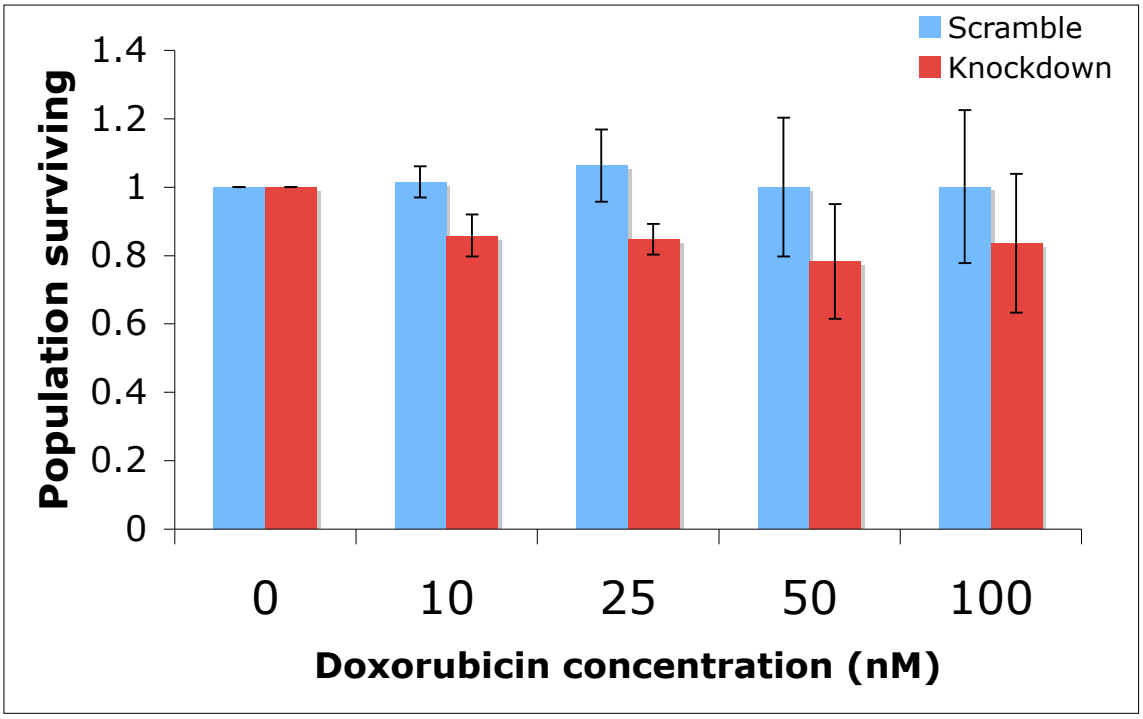

Figure 11: Chemoresistance studies of CD68 knockdown.
Preliminary testing of breast cancer cell lines 231-Scramble compared to 231-CD68-knockdown reveal a trend of chemoresistance when CD68 is intact. Future studies will incidate if the knockdown or suppression of CD68 can render breast cancer more susceptible to chemotherapies

IL-11 PRODUCED BY BREAST CANCER CELLS AUGMENTS OSTEOCLASTOGENESIS BY SUSTAINING THE POOL OF OSTEOCLAST PROGENITOR CELLS

BMC Cancer 2013, 13:16 doi:10.1186/1471-2407-13-16

By

ERIN M MCCOY, HUIXIAN HONG, HAWLEY C PRUITT, XU FENG

Article under revision for BMC Cancer

Errata corrected and format adapted for dissertation

Abstract

Interleukin (IL)-11, a cytokine produced by breast cancer, has been implicated in breast cancer-induced osteolysis (bone destruction) but the mechanism(s) of action remain controversial. Some studies show that IL-11 is able to promote osteoclast formation independent of the receptor activator of NF-κB ligand (RANKL), while others demonstrate that IL-11 can induce osteoclast formation by inducing osteoblasts to secrete RANKL. This work aims to further investigate the role of IL-11 in metastasis-induced osteolysis by addressing a new hypothesis that IL-11 exerts effects on osteoclast progenitor cells.

To address the precise role of breast cancer-derived IL-11 in osteoclastogenesis, we determined the effect of breast cancer conditioned media on osteoclast progenitor cells and found that freshly isolated murine bone marrow cells cultured in the presence of breast cancer conditioned media for 6 days gave rise to a population of cells which were able to form osteoclasts upon treatment with RANKL and M-CSF. Moreover, a neutralizing anti-IL-11 antibody significantly inhibited the ability of breast cancer conditioned media to promote the development and/or survival of osteoclast progenitor cells. Similarly, we found that recombinant IL-11 was able to sustain a population of osteoclast progenitor cells as well. However, IL-11 was unable to exert any effect on osteoclast survival, induce osteoclastogenesis independent of RANKL, or promote osteoclastogenesis in suboptimal RANKL conditions.

Our data indicate that a) IL-11 plays an important role in osteoclastogenesis by stimulating the development and/or survival of osteoclast progenitor cells and b) breast cancer may promote osteolysis in part by increasing the pool of osteoclast progenitor cells via tumor cell-derived IL-11. However, given the heterogeneous nature of the bone marrow cells, the precise mechanism by which IL-11 treatment gives rise to a population of osteoclast progenitor cells warrants further investigation.

Introduction

Breast cancer is the second leading cause of cancer deaths in women in the United States and this tumor frequently metastasizes to bone. Upon arriving in bone, breast cancer cells disrupts the normal bone remodeling by increasing bone resorption, leading to several serious clinical complications including life-threatening hypercalcemia, spinal cord compression, fractures, and extreme bone pain, which result in a

significantly decreased quality of life [1, 2]. Bone metastases have also been hypothesized to serve as reservoirs for breast cancer to metastasize to other tissues, such as the lung, liver, lymph node, or brain [3]. Thus, breast cancer patients with bone metastases often have a poor prognosis [4].

Breast cancer cells have been shown to promote bone resorption by enhancing osteoclast formation and function via a number of factors derived from the tumor including M-CSF, transforming growth factor (TGF)-β, tumor necrosis factor α, insulin-like growth factor II, parathyroid hormone related peptide, IL-1, IL-6 and IL-11 [2, 5-7]. IL-11 is a member of the IL-6 family that recruits a homodimer of gp130, a promiscuous 130kDa β subunit, after binding to their own non-signaling ligand-specific receptor, IL11R [8, 9]. IL-11 is produced by a variety of stromal cells, including fibroblasts, epithelial cells, and osteoblasts and has a variety of functions, including being involved in multiple aspects of hematopoiesis, inhibition of adipocytogenesis, altering neural phenotype, stimulating tissue fibrosis, minimizing tissue injury, and regulating function of chondrocytes, synoviocytes and B cells [10]. Apart from contributing to inflammation, gp130 signaling cytokines also function in the maintaining bone homeostasis.

Cancer cells have been shown to directly produce IL-11 and to stimulate osteoblasts to secrete IL-11 [11], which in turn is known to suppress the activity of osteoblasts [12]. It has been shown that breast cancer cell lines produce IL-11 [13] and that forced over-expression in cell lines increases tumor burden and osteolytic lesions in an *in vivo* bone metastasis model [5]. Moreover, human breast cancer tumors expressing IL-11 have higher rates of bone metastasis occurrences [3]. Taken together, these observations support the notion that IL-11 plays an important role in breast cancer-induced osteolysis.

Using a knockout mouse model for IL-11, the cytokine was determined to be required for normal bone turnover, with the knockout mice exhibiting increased bone mass as a result of a reduction in osteoclast differentiation [14]. IL-11 has been proposed to stimulate osteoclastogenesis independent of RANKL in one study [15], whereas another study showed that IL-11 did not induce osteoclastogenesis unless marrow cells were co-cultured with calvaria cells [16]. Similarly, other groups argue that IL-11 stimulates osteoblasts to secrete RANKL and/or proteinases [17, 18]. Thus, while a functional role of IL-11 in the osteoclastogenic process has been well established, the molecular and cellular mechanisms by which IL-11 promotes osteoclast differentiation and function warrant further investigation. Given the known role of IL-11 in hematopoiesis [10], we hypothesize that IL-11 may exert effects on osteoclast progenitor cells.

In the current study, we further characterize the role of IL-11 in supporting osteoclast formation, function and survival. Our data indicate that IL-11 promotes osteoclastogenesis primarily by increasing the pool of osteoclast progenitor cells. Consistently, we have also found that MDA-MB-231 conditioned media were able to support a population of bone marrow cells, which are capable of differentiating into osteoclasts. These findings have not only provided a better understanding of the mechanism by which IL-11 exerts its impact on osteoclast biology but have also suggested a new concept that breast cancer may also promote osteoclast formation by targeting osteoclast progenitor cells.

Materials and Methods

Chemicals and reagents

Chemicals were purchased from Sigma (St. Louis, MO) unless indicated otherwise. Recombinant GST-RANKL was purified as described previously [19]. Recombinant mouse M-CSF (rM-CSF) (416-ML-010) and IL-11 (418-ML-005) were obtained from R&D Systems (Minneapolis, MN). Neutralizing anti-human IL-11 antibody (AB-218-NA) and normal goat IgG control antibody (AB-108-C) were also obtained from R&D Systems.

Animals

C57BL/6 mice were purchased from Harlan Industries (Indianapolis, IN). Mice were maintained, and the experiments performed in accordance with the regulations of the University of Alabama at Birmingham (UAB) institutional animal care and use committee (IACUC).

In vitro osteoclastogenesis assays

Breast cancer conditioned α-MEM was prepared by growing the human breast cancer line MDA-MB-231 to confluence, changing media to α-MEM plus 10% inactivated fetal bovine serum (iFBS), and collecting conditioned media after 24 hours. To generate osteoclasts from breast cancer conditioned media- dependent precursors, cells from the bone marrow cavities of the femur and tibia from C56BL/6 mice less than eight weeks of age were used. The bone marrow flushes were maintained in α-MEM for 24 hours at 37°C, in 7% CO_2, and then cultured in breast cancer conditioned α-MEM or regular α-MEM supplemented with

10% iFBS. Media were changed every 3 days, and after 6 days, cells from the breast cancer conditioned α-MEM pretreated bone marrow flushes were plated in tissue-culture treated dishes at varying densities as indicated specifically in each experiment and treated with rM-CSF (10 ng/ml) and RANKL (100 ng/ml) for 4-6 days to form osteoclasts. Separately, IL-11 neutralizing antibody (5 ug/ml) was added to bone marrow flushes in 20% MDA-MB-231 breast cancer conditioned media, and surviving cells counted at days 3, 4, 5, and 6.

To generate osteoclasts from IL-11-dependant precursors, bone marrow flushes were maintained in α-MEM for 24 hours at 37°C, in 7% CO_2, and then cultured in α-MEM with the presence of IL-11 (10ng/ml) or equal volumes of PBS containing 0.01% bovine serum albumin. After 6 days, cells from the IL-11 pretreated bone marrow flushes were plated in tissue-culture treated dishes at varying densities as indicated specifically in each experiment and treated with rM-CSF (10 ng/ml) and RANKL (100 ng/ml) for 4-6 days to form osteoclasts. Separately, IL-11 neutralizing antibody (2ug/ml) was added to bone marrow flushes in α-MEM with the presence of IL-11 (10ng/ml), and surviving cells counted at days 3, 4, 5, and 6.

For IL-11 mechanistic studies of osteoclastogenesis, BMMs were isolated from marrow flushes of the long bones of 4–8-week-old C57BL/6 mice and were maintained in α-minimal essential medium (α-MEM) for 24 hours at 37°C, in 7% CO_2, before separation with Ficoll gradient. To generate osteoclasts from BMMs, following Ficoll gradient separation, 1×10^5 or 5×10^4 cells, respectively, were plated in either 24-well or 48-well tissue culture plates. Cells were cultured in the presence of different concentrations and combinations of rM-CSF, RANKL, and IL-11 as indicated in individual experiments. The osteoclastogenesis cultures were stained for tartrate resistant acid phosphatase (TRAP) activity with a Leukocyte Acid Phosphatase kit (387-A) from Sigma. All assays were performed in triplicate and repeated at least three times. A representative view from each condition is shown.

In vitro bone resorption assays

5×10^4 BMMs were plated on bovine cortical bone slices in 24-well plates, and the cultures were treated with rM-CSF (10 ng/ml) and RANKL (100 ng/ml), or with IL-11, rM-CSF, or RANKL as detailed in each experiment. Cultures were maintained for 9 days to allow for bone resorption and then cells were removed

from the bone slices with 0.25 M ammonium hydroxide and mechanical agitation. Bone slices were then subjected to scanning electron microscopy (SEM) using a Philips 515 SEM (Materials Engineering Department, University of Alabama at Birmingham). The percentage of the resorbed area was determined using ImageJ analysis software obtained from the National Institutes of Health.

Statistical analysis

Osteoclastogenesis data are expressed as mean ± standard error (SE) of numbers of TRAP-positive cells. Cell viability assays are expressed as mean ± SE of numbers of viable cells on each day counted. Statistical significance was determined using Student's *t* test, and *p* values less than 0.05 were considered significant.

Results

Breast cancer conditioned media are capable of supporting the development and/or survival of osteoclast progenitor cells

Given that previous studies showed that human breast cancer cell line MDA-MB-231 expresses IL-11 [13, 20], we investigated whether MDA-MB-231 conditioned media are able to promote the development and/or survival of osteoclast progenitor cells in the whole bone marrow. Bone marrow flushes were cultured in regular media or breast cancer conditioned media, prepared from human breast cancer cell line MDA-MB-231, for varying number of days. As shown in Figure 1A, the cultures in the conditioned media had more cells than those in regular media. More importantly, we found that the cells from the cultures in the conditioned media were capable of forming functional osteoclasts in response to M-CSF and RANKL treatment, and that osteoclast number and morphology was dependent on density of progenitor cells plated (Figure 1B-C). These data indicate that breast cancer cells produce factors, presumably including IL-11, which are capable of stimulating the development and/or survival of osteoclast progenitor cells. Thus, these data suggest that breast cancer may enhance the extent of osteoclastogenesis by augmenting the pool of osteoclast progenitor cells.

IL-11 promotes the development and/or survival of osteoclast progenitor cells

Next, we examined whether recombinant IL-11 is able to replicate the results seen with breast cancer conditioned media (Figure 1). To do so, we cultured bone marrow flushes in α-MEM with or with IL-11 (10ng/ml) for varying number of days. While most cells died in the cultures supplemented with vehicle (PBS), a significant number of cells remained alive and healthy in those treated with IL-11 (Figure 2A). Moreover, we found that the IL-11-dependent bone marrow cells were able to differentiate into functional osteoclasts in response to M-CSF and RANKL treatment (Figure 2B-C). Again, the osteoclast number and morphology was dependent on the density of the cells plated. These findings demonstrate that IL-11 is able to promote the development and/or survival of osteoclast progenitor cells.

IL-11 neutralizing antibody reduces breast cancer conditioned media's ability to promote the development and/or survival of osteoclast progenitor cells

To determine whether IL-11 is the predominant factor derived from MDA-MB-231 cells that stimulate the development and/or survival of osteoclast progenitors, we repeated the experiment shown in Figure 1 with an anti-human IL-11 neutralizing antibody. We first validated the neutralizing capability of the commercial IL-11 neutralizing antibody by culturing bone marrow flushes in α-MEM containing IL-11 (10ng/ml) with control IgG or IL-11 neutralizing antibody (Figure 3A). Next, to determine the lowest optimal concentration of breast cancer conditioned media that could facilitate the development and/or survival of osteoclast progenitors, bone marrow flush cells were cultured in α-MEM or α-MEM with increasing concentrations of MDA-MB-231 conditioned media. There was no significant difference between using 20% or 40% breast cancer conditioned media, but both supported significantly more cells than 0, 5, or 10% (Figure 3B). Finally, bone marrow flushes cultured in 20% MDA-MB-231 breast cancer conditioned media were subjected to the IL-11 neutralizing antibody (5ug/ml) (Figure 3C). Our data demonstrated that the neutralizing IL-11 antibody significantly reduced the ability of the conditioned media to promote the development and/or survival of osteoclast progenitor cells, indicating that IL-11 is the predominant factor derived from MDA-MB-231 cells that stimulate the development and/or survival of osteoclast progenitors. This finding further suggests that certain breast cancers may increase the extent of osteoclastogenesis by expanding the pool of osteoclast progenitor cells via tumor-derived IL-11.

IL-11 does not affect osteoclast survival

Given that our data have shown that IL-11 sustains a population of cells containing osteoclast precursors, we extended our study to address whether IL-11 exerts any effect on the survival of mature osteoclasts. Toward this end, we treated BMMs with rM-CSF and RANKL for 4 days to promote osteoclast formation. Once osteoclasts formed, we removed the media containing rM-CSF and RANKL and added IL-11 or PBS (vehicle) to the cultures, which were continued for 8 additional days to determine IL-11's effect on survival. The representative TRAP staining images of the osteoclast cultures treated with IL-11 for 8 days are shown in Figure 4A, while survived osteoclasts in the cultures treated with IL-11 were quantified at day 6 and day 8 (Figure 4B). At both day 6 and day 8, there was no statistically significant difference in osteoclast survival between the IL-11-treated cultures and the PBS-treated control cultures. Morphologically, the cultures also looked very similar. The data indicate that IL-11 does not play a role in osteoclast survival.

IL-11 is unable to stimulate osteoclastogenesis in the absence of RANKL

The role of IL-11 in osteoclastogenesis remains unclear; while one study demonstrated that IL-11 is able to stimulate osteoclastogenesis independent of RANKL [15], another group showed that IL-11 cannot induce osteoclastogenesis unless marrow cells are co-cultured with calvaria cells [16], which may serve as a source of RANKL. To further investigate the role of IL-11 in osteoclastogenesis, we examined IL-11's ability to induce osteoclastogenesis in the absence of RANKL. To this end, IL-11 was added at different concentrations (5, 10, and 20 ng/ml) along with 10ng/ml rM-CSF to bone marrow macrophages, and then stained for TRAP activity on day 6 (Figure 5A). While confluent TRAP positive multinucleated osteoclasts were formed under control conditions of RANKL (100ng/ml) and rM-CSF (10ng/ml), none of the concentrations of IL-11 were sufficient to induce osteoclastogenesis in the absence of RANKL in tissue culture dishes. Furthermore, we performed bone resorption assays to determine whether IL-11 can promote osteoclast formation on bone slices. Our data reveal that IL-11 is incapable of stimulating functional osteoclasts on bone slices, as shown by the lack of resorption pits on the IL-11 treated bone slices (Figure 5B). To further address whether higher doses of IL-11 can promote osteoclastogenesis in the absence of RANKL, we repeated the experiment with 200 ng/ml IL-11. The data indicate that, even at concentrations

as high as 200 ng/ml, IL-11 is still unable to stimulate osteoclastogenesis (Figure 5C). These findings indicate that IL-11 cannot promote osteoclast differentiation independent of RANKL.

IL-11 cannot stimulate osteoclastogenesis even with low levels of RANKL

We and others have demonstrated that although several cytokines such as IL-1 and tumor necrosis factor α (TNF-α) cannot promote osteoclastogenesis in the absence of RANKL, they are able to do so in the presence of permissive levels of RANKL [21-26]. So we next investigated if IL-11 can stimulate osteoclastogenesis in the presence of low levels of RANKL. BMMs were cultured with rM-CSF (10ng/ml) plus RANKL (10ng/ml) with or without IL-11 (10ng/ml) for 6 days in tissue culture dishes and then stained for TRAP activity (Figure 6A). We found that IL-11 was not able to induce osteoclastogenesis with low levels of RANKL in tissue culture dishes. The assay was repeated on bone slices and the bone resorption assays showed that IL-11 failed to promote the formation of functional osteoclasts on bone slices in the presence of 10ng/ml RANKL, as shown by the lack of resorption pits (Figure 6B). To further address whether higher doses of IL-11 can promote osteoclastogenesis in the low levels of RANKL, we repeated the experiment with 200ng/ml IL-11. The data indicate that, even at concentrations as high as 200ng/ml, IL-11 is still unable to stimulate osteoclastogenesis in the presence of low levels of RANKL (Figure 6C). Taken together, we conclude that IL-11, unlike IL-1 and TNF-α, is incapable of stimulating osteoclastogenesis even in the presence of low levels of RANKL.

IL-11 is incapable of stimulating osteoclastogenesis from RANKL-primed BMMs

We and others have also shown that IL-1 and TNF-α can also promote osteoclastogenesis from RANKL-primed BMMs [21-26]. To determine whether IL-11 can function in osteoclastogenesis in this manner, BMMs were pretreated for 24 hours with or without RANKL in the presence of rM-CSF in tissue culture dishes or on bone slices. After 24 hours, the media was removed and replaced with media containing rM-CSF with either IL-11 or RANKL, and the cultures were continued for 4 days (Figure 7). The assays demonstrated that IL-11 was unable to stimulate osteoclastogenesis from RANKL-primed BMMs in tissue culture dishes (Figure 7, top row) or on bone slice (Figure 7, B and C).

Discussion

Since the initial study showing the expression of IL-11 in breast tumor tissues more than 25 years ago [27], numerous investigations have been subsequently undertaken to address the regulation and pathological significance of IL-11 expression in breast cancer and, in particular, in the tumor-induced osteolysis [5, 13, 28-34]. Collectively, these studies have led to two important observations: a) IL-11 is not only expressed in a significant number of breast cancers but also has the potential to serve as a prognostic factor in human breast cancer, and b) IL-11 plays an important role in breast cancer-mediated osteolysis by promoting osteoclastogenesis and bone resorption. Notably, several studies have demonstrated that breast tumor cells can also target osteoblasts to stimulate their production of IL-11 [11, 17], further increasing IL-11 concentrations in the bone microenvironment. Therefore, elucidation of the molecular mechanism by which IL-11 increases osteoclastogenesis and bone resorption in breast cancer bone metastasis may help guide development of effective drugs and/or therapeutic regimens for preventing and treating breast cancer-induced osteolysis.

Early studies on the role of IL-11 in osteoclast formation and function involved the use of the co-culture system containing bone marrow cells and calvarial osteoblasts [16, 35]; the key finding of these early investigations was that IL-11-mediated osteoclastogenesis requires the presence of osteoblasts, but the precise reason for the dependence of IL-11-mediated osteoclastogenesis on osteoblasts was not fully understood. After the discovery of the RANKL/RANK/OPG system in the late 1990s, it then became clear that osteoblasts in the co-culture system primarily serve as a source of RANKL and IL-11 stimulates osteoblasts to produce RANKL [36, 37]. This led to the notion that IL-11 can promote osteoclastogenesis indirectly by stimulation osteoblast production of RANKL. On the other hand, it was shown that osteoclasts express IL-11R [35], suggesting that IL-11 may also directly target osteoclasts and/or its precursors to regulate osteoclast formation and/or function. Intriguingly, one study demonstrated that IL-11 directly target osteoclast precursors to stimulate osteoclastogenesis and it does so independent of RANKL [15]. However, this finding is inconsistent with the early studies showing that IL-11-mediated osteoclastogenesis requires the presence of osteoblasts, which is a known source of RANKL.

In this work, we independently carried out a series of *in vitro* studies to further address the role of IL-11 in osteoclastogenesis. First we determined that the conditioned media of MDA-MB-231, a breast cancer

cell line expressing IL-11 [13, 20], gave rise to a population of cells which can form osteoclasts in response to RANKL and M-CSF treatment (Figure 1), indicating that IL-11 may play an important role in osteoclastogenesis by regulating the development and/or survival of osteoclast progenitor cells. Because the MDA-MB-231 also secrete other factors that play a role in osteoclastogenesis it was necessary to look specifically at IL-11 function. Importantly, the ability of the breast cancer conditioned media to generate a population of osteoclast progenitor cells was significantly inhibited by a neutralizing anti-IL-11 antibody (Figure 3). These findings suggest that tumor-derived IL-11 may increase the extent of osteoclastogenesis by promoting the development of a population of osteoclast progenitor cells. To verify the specificity of IL-11, we found that culturing of murine bone marrow cells with IL-11 for 6 days is able to give rise to a pool of osteoclast progenitor cells (Figure 2).

We then investigated other ways that IL-11 may play a role in osteoclastogenesis. We found that IL-11 does not exert any effect on osteoclast survival (Figure 4). We then examined if IL-11 is able to promote osteoclast formation in the absence of RANKL and our data demonstrate that IL-11 cannot induce osteoclastogenesis in tissue culture dishes or on bone slices in the absence of RANKL (Figure 5). We and others have demonstrated that while IL-1 and TNF-α cannot promote osteoclastogenesis in the absence of RANKL, they can do so with suboptimal levels of RANKL or from RANKL-pretreated BMMs [21-26]. As such, we then investigated whether IL-11 can act in a similar manner. Our data show that IL-11 is not able to promote osteoclastogenesis in the presence of suboptimal levels of RANKL (Figure 6) or from RANKL-pretreated BMMs (Figure 7).

Based on these new findings and those reported previously [16, 34, 36, 37], we propose that IL-11-expressing breast cancer cells cause increased osteoclast formation and bone resorption by two distinct mechanisms: a) the tumor cells produce IL-11 which in turn stimulate the production of RANKL by stromal cells/osteoblasts in the bone microenvironment, and b) tumor cell-derived IL-11 also augments the pool of osteoclast progenitor cells to increase the extent of osteoclastogenesis.. Therefore, our work has led to a better understanding of the action of IL-11 in breast cancer-induced osteolysis. However, the precise mechanism by which IL-11 promotes the development of a population of osteoclast progenitor cells remains unclear. While it is possible that IL-11 does so by stimulating the differentiation, proliferation and/or survival of osteoclast progenitor cells, this cytokine may exert the impact on osteoclast progenitor

cell population indirectly through other cell types in the bone marrow. Further studies are needed to elucidate how exactly IL-11 promote the development of a pool of osteoclast progenitor cells.

Moreover, our new data may help guide the development of better therapeutic regimens for preventing and treating breast cancer-induced osteolysis. Particularly, denosumab, a humanized anti-RANKL developed by Amgen Inc, has been approved by the FDA to treat breast cancer-mediated osteolysis. For IL-11 positive tumors, denosumab may be effective only in blocking the RANKL-dependent action of IL-11. In contrast, it is likely that an efficient inhibition of IL-11 can block the IL-11-mediated increase of the pool of osteoclast progenitor cells as well as the RANKL-dependent pathway, thus having the potential to give rise to better efficacy. Future animal model studies need to be undertaken to address the therapeutic potential of targeting IL-11.

Conclusion

In conclusion, these studies demonstrate that IL-11 exerts its effect on osteoclastogenesis primarily by targeting osteoclast progenitor cells, specifically through promoting the development and/or survival of osteoclast progenitor cells. Moreover, we show that MDA-MB-231 breast cancer cells are able to stimulate the development and/or survival of osteoclast progenitor cells and IL-11 is the predominant factor derived from MDA-MB-231 cells that is responsible. This suggests that some breast cancers may increase the extent of osteoclastogenesis by augmenting the pool of osteoclast progenitor cells via tumor-derived IL-11. Importantly, these findings have not only provided a better understanding of the role of IL-11 in breast cancer bone metastasis but also laid a foundation for future investigations to address therapeutic targeting of IL-11 for treating and preventing breast cancer induced osteolysis.

List of abbreviations

α-MEM: α-minimal essential medium; BMMs: Bone marrow macrophages; IL-1: Interleukin 1; IL-6: Interleukin 6; IL-11: Interleukin 11; M-CSF: Monocyte/macrophage-colony stimulating factor; RANKL: Receptor activator of nuclear factor κB ligand; rM-CSF: Recombinant M-CSF; SEM: Scanning electron microscopy; TRAP: Tartrate resistant acid phosphatase; TNF-α: Tumor necrosis factor α

Authors' contributions

All authors read and approved the final manuscript. EM developed the idea, performed the experiments, analyzed the data, and prepared the manuscript. HH and HCP provided technical assistance. XF initially conceived the idea, and participated in the experimental design and manuscript preparation.

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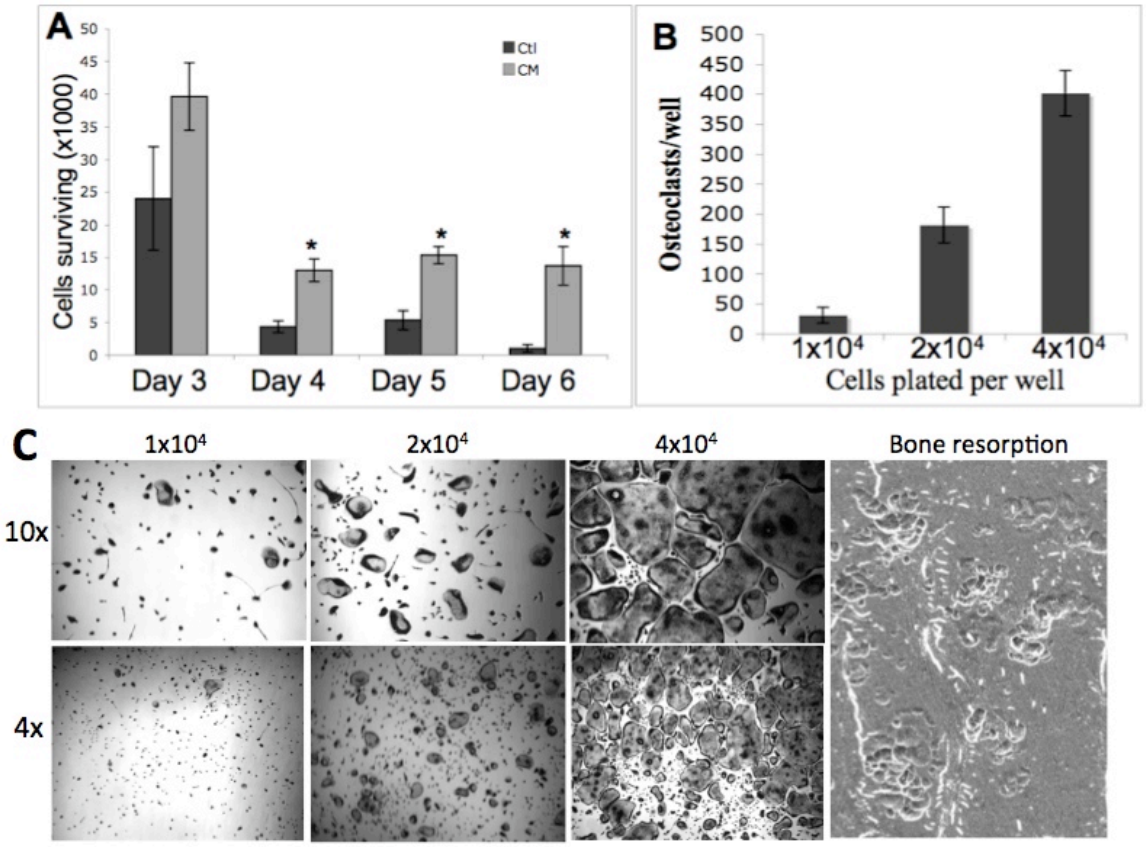

Figure 1 Breast cancer conditioned media is capable of stimulating the development and/or survival of osteoclast progenitor cells. (A) Bone marrow flushes were cultured in α-MEM (Ctl) or MDA-MB-231 conditioned α-MEM (CM) and surviving cells were counted at days 3, 4, 5, and 6. Data are expressed as a mean +/- S.E. *, $p<0.02$. (B) On day 6, cells from the culture in breast cancer conditioned α-MEM were then seeded into 48 well plates at 1×10^4, 2×10^4, or 4×10^4 cells per well and treated with 100ng/ml RANKL and 10ng/ml rM-CSF. Quantification of the osteoclastogenesis assays is shown in mean number of multinucleated TRAP-positive cells (>3 nuclei) per well. (C) Each condition had three replicates (wells) and was repeated 4 times. A representative area of the culture from each condition is shown (4× and 10× magnification). A separate set of cultures was continued 4 additional days to perform bone resorption assays. Resorption pits were then visualized by SEM. Magnification by SEM was 200x.

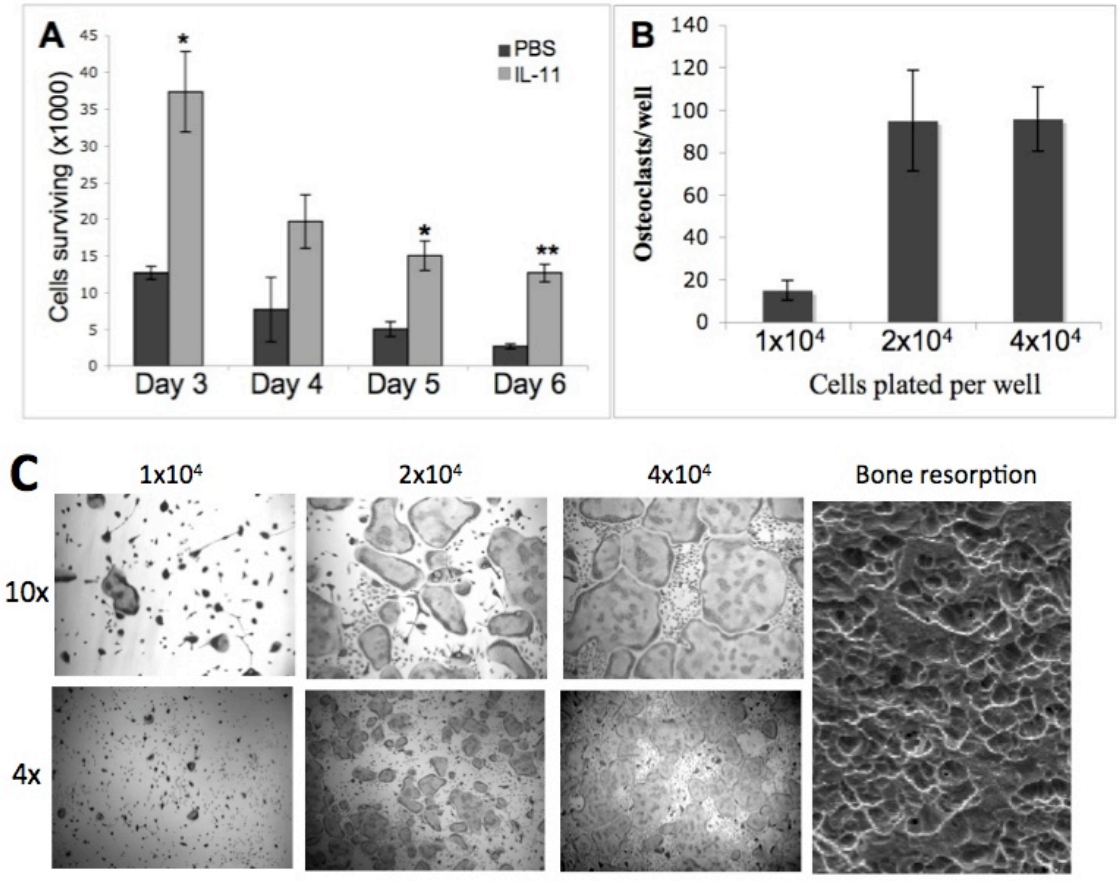

Figure 2 IL-11 is able to promote the development and/or survival of osteoclast progenitor cells. (A) Bone marrow flush cultured in α-MEM containing IL-11 (10ng/ml) or equal volume of vehicle (PBS) and remaining surviving cells were counted at day 3, 4, 5, and 6. Data are expressed as a mean +/- S.E. *, $p< 0.02$, **, $p<0.002$. (B) On day 6, IL-11-dependent bone marrow cells were then seeded into 48 well plates at 1×10^4, 2×10^4, or 4×10^4 cells per well and treated with 100ng/ml RANKL and 10ng/ml rM-CSF. Quantification of the osteoclastogenesis assays is shown in mean number of multinucleated TRAP-positive cells (>3 nuclei) per well. (C) Each condition had three replicates (wells) and was repeated 4 times. A representative area of the culture from each condition is shown (4× and 10× magnification). A separate set of cultures was continued 4 additional days to perform bone resorption assays. Resorption pits were then visualized by SEM. Magnification by SEM was 200x.

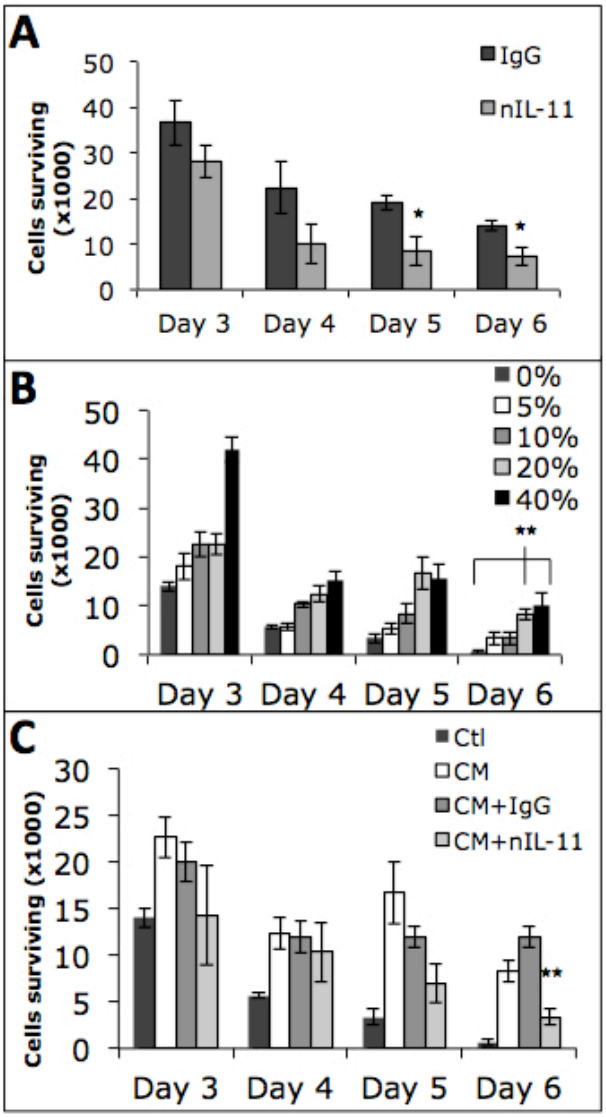

Figure 3 IL-11 neutralizing antibody reduces breast cancer conditioned media's ability to give rise to osteoclast progenitors. (A) Bone marrow flushes were cultured in α-MEM containing IL-11 (10ng/ml) with control IgG (IgG) or IL-11 neutralizing antibody (nIL-11, 2ug/ml) for 6 days. Surviving cells were counted at day 3, 4, 5, and 6. (B) Bone marrow flushes were cultured in α-MEM (0%) or α-MEM with increasing concentrations (5%, 10%, 20% or 40%) of MDA-MB-231 conditioned media for 6 days. Surviving cells were counted at day 3, 4, 5, and 6. (C) Bone marrow flushes were cultured in α-MEM (ctl), 20% MDA-MB-231 conditioned α-MEM (CM), 20% MDA-MB-231 conditioned α-MEM with control IgG (CM+IgG, 5ug/ml), or 20% MDA-MB-231 conditioned α-MEM with IL-11 neutralizing antibody (CM+nIL-11, 5ug/ml). Surviving cells were counted at day 3, 4, 5, and 6. All data were repeated independently three times and are expressed as a mean +/- S.E, *, $p<0.05$; **, $p<0.004$.

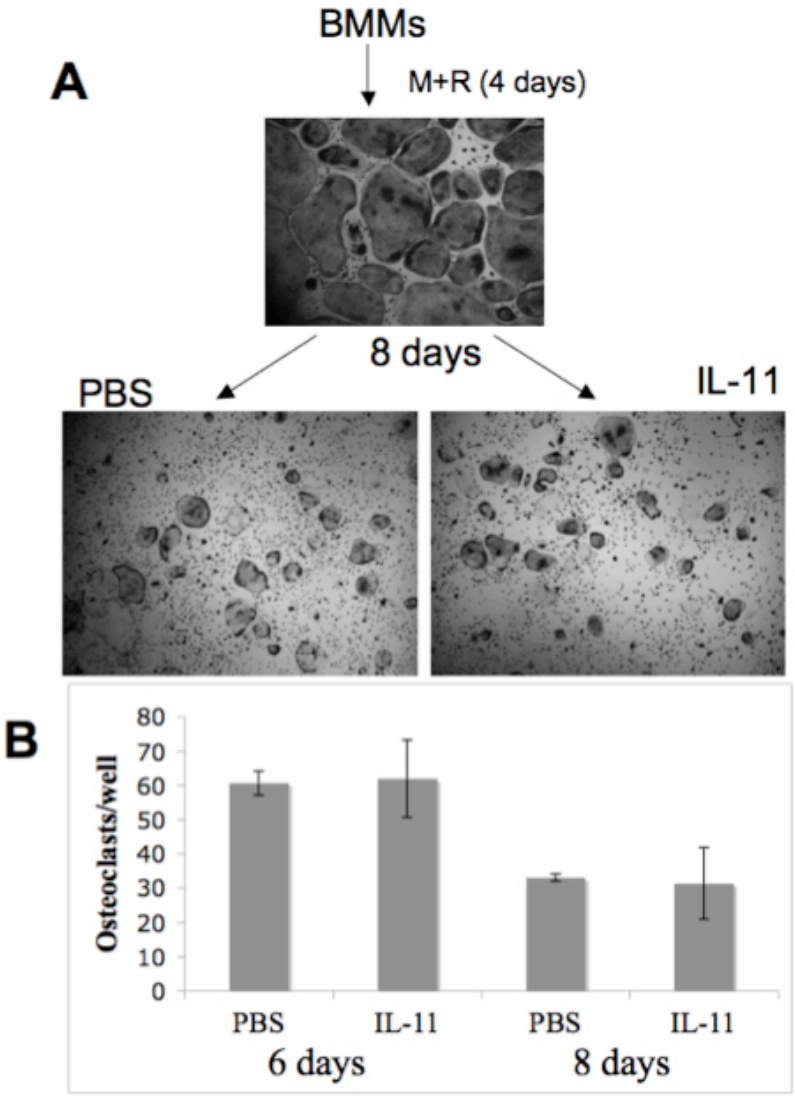

Figure 4 IL-11 does not affect osteoclast survival. (A) BMMs were cultured with rM-CSF (10ng/ml) plus RANKL (100ng/ml) for 4 days until sufficient osteoclasts were formed and then cultured with rM-CSF (10ng/ml) and RANKL (100ng/ml) with vehicle (PBS) or IL-11 (10g/ml) for 8 days in tissue culture dish. The cultures were then stained for TRAP activity. Each condition had three replicates (wells) and was repeated 4 times. A representative area of the culture from each condition is shown. (B) Quantification of the osteoclastogenesis assays is shown in mean number of multinucleated TRAP-positive cells (>3 nuclei) per well. Bars show averages ± S.D.

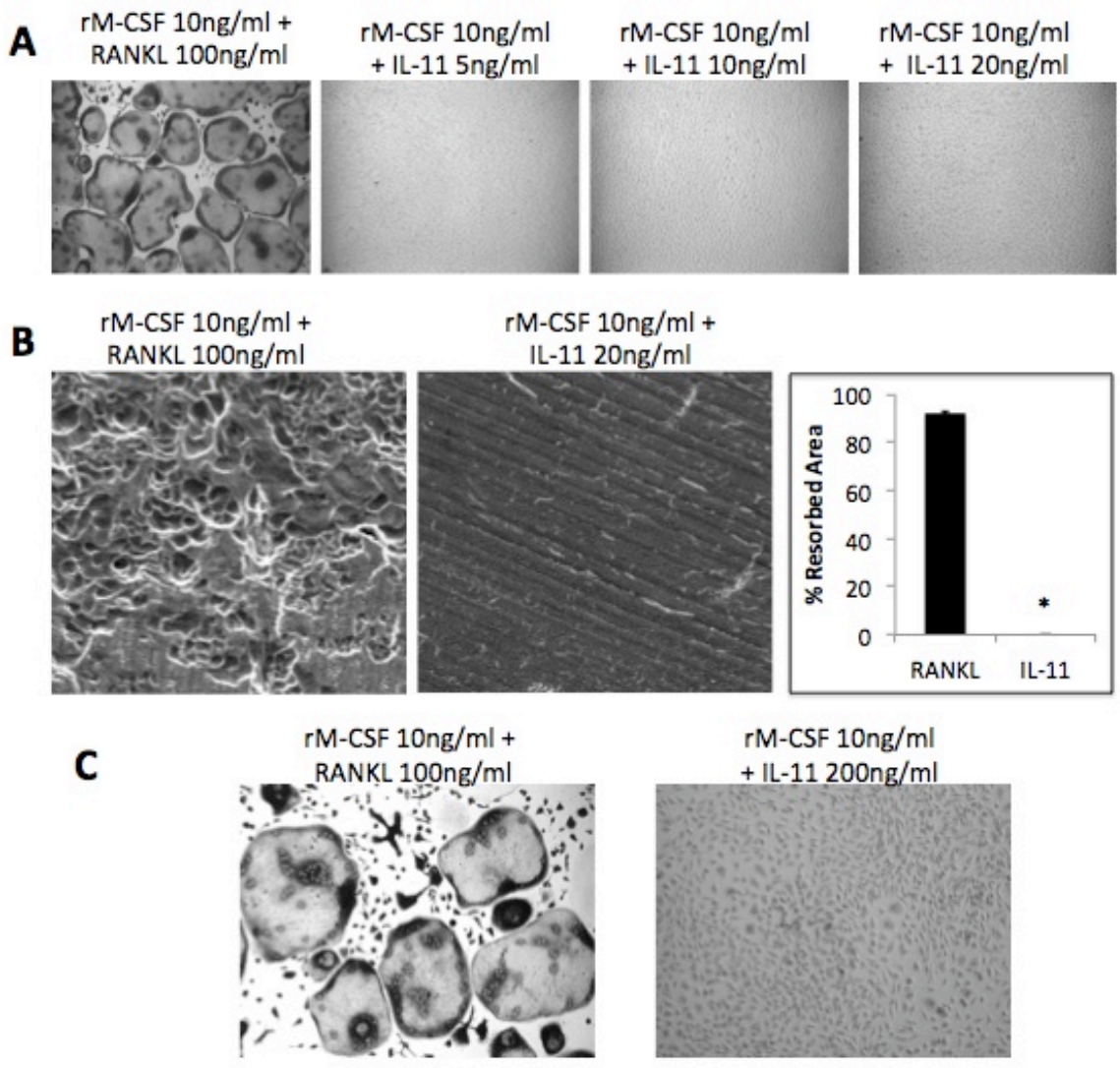

Figure 5 IL-11 fails to stimulate osteoclastogenesis in the absence of RANKL. (A) BMMs were cultured with rM-CSF (10ng/ml) plus RANKL (100ng/ml) as control or M-CSF (10ng/ml) plus IL-11 (5, 10 or 20ng/ml) for 6 days in tissue culture dish. The cultures were then stained for TRAP activity. Each condition had three replicates (wells) and was repeated 4 times. A representative area of the culture from each condition is shown. (B) BMMS on bone slices were treated with rM-CSF (10 ng/ml) plus RANKL (100 ng/ml) as control or rM-CSF (10 ng/ml) plus IL-11 (20 ng/ml) for 9 days. Resorption pits were visualized by SEM. Magnification by SEM was 200x. Each resorption assay had two replicates (bone slices). Quantification of the bone resorption assays is shown, bars shown averaged ± S.E. *, $p<0.0001$ (C) BMMs were cultured with rM-CSF (10ng/ml) plus RANKL (100ng/ml) as control or M-CSF (10ng/ml) plus IL-11 (200ng/ml) for 6 days in a tissue culture dish. The cultures were then stained for TRAP activity. Each condition had three replicates (wells) and was repeated 3 times. A representative area of the culture from each condition is shown.

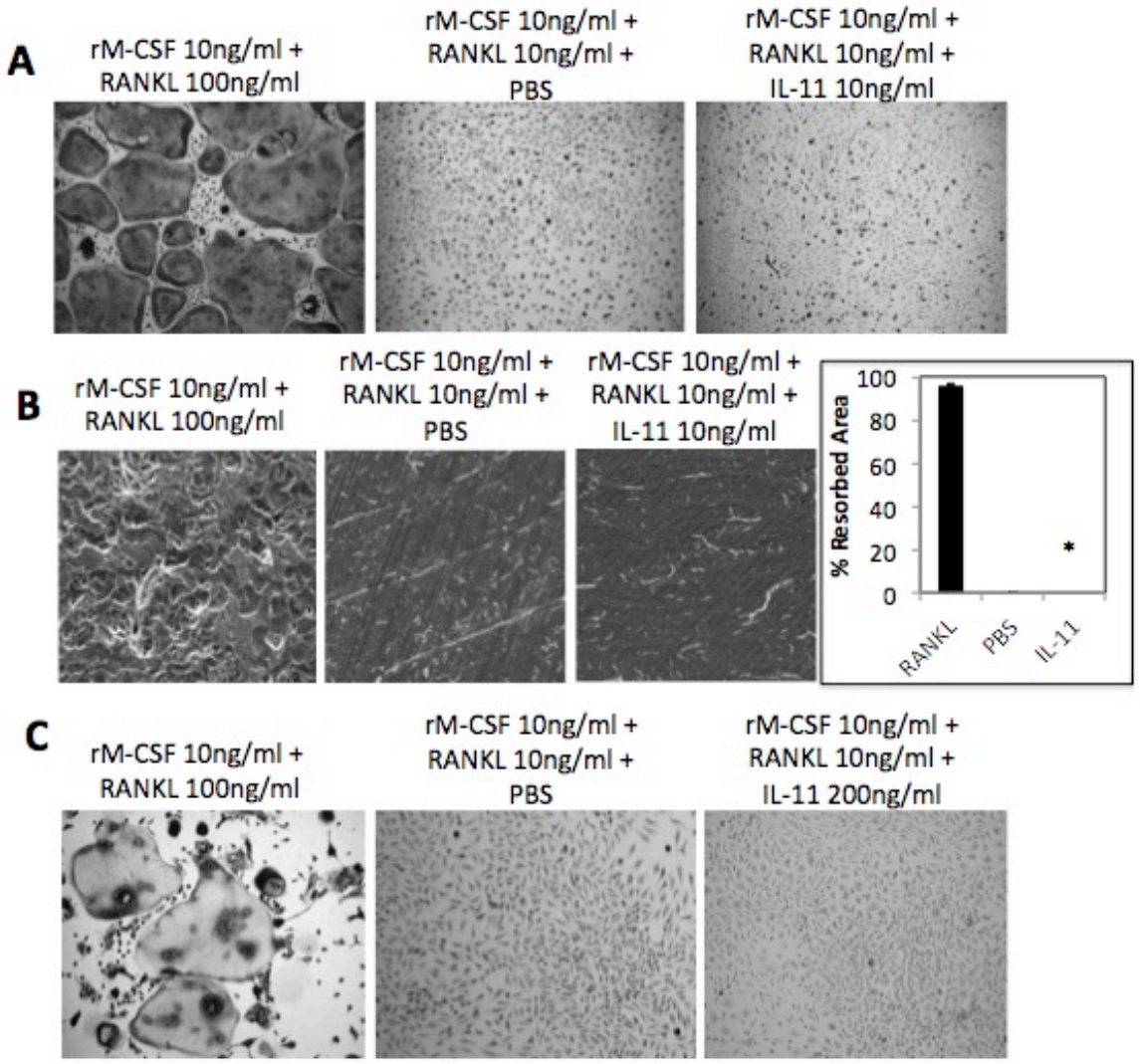

Figure 6 IL-11 fails to stimulate osteoclastogenesis even with permissive level of RANKL. (A) BMMs were cultured with rM-CSF (10 ng/ml) plus RANKL (10 ng/ml) with or without IL-11 (10 ng/ml) for 6 days in tissue culture dish. The cultures were then stained for TRAP activity. Each condition had three replicates (wells) and was repeated 4 times. A representative area of the culture from each condition is shown. (B) BMMs on bone slices were treated with the same conditions, but cultured for 9 days and resorption pits were then visualized by SEM. Magnification by SEM was 200x. Each resorption assay had two replicates (bone slices) Quantification of the bone resorption assays is shown, bars shown averaged ± S.E. *, $p<0.0001$ (C) BMMs were cultured with rM-CSF (10 ng/ml) plus RANKL (100 ng/ml) as control or rM-CSF (10 ng/ml) with sub-optimal levels of RANKL (10 ng/ml) with or without IL-11 (200 ng/ml) for 6 days in a tissue culture dish. The cultures were then stained for TRAP activity. Each condition had three replicates (wells) and was repeated 3 times. A representative area of the culture from each condition is shown.

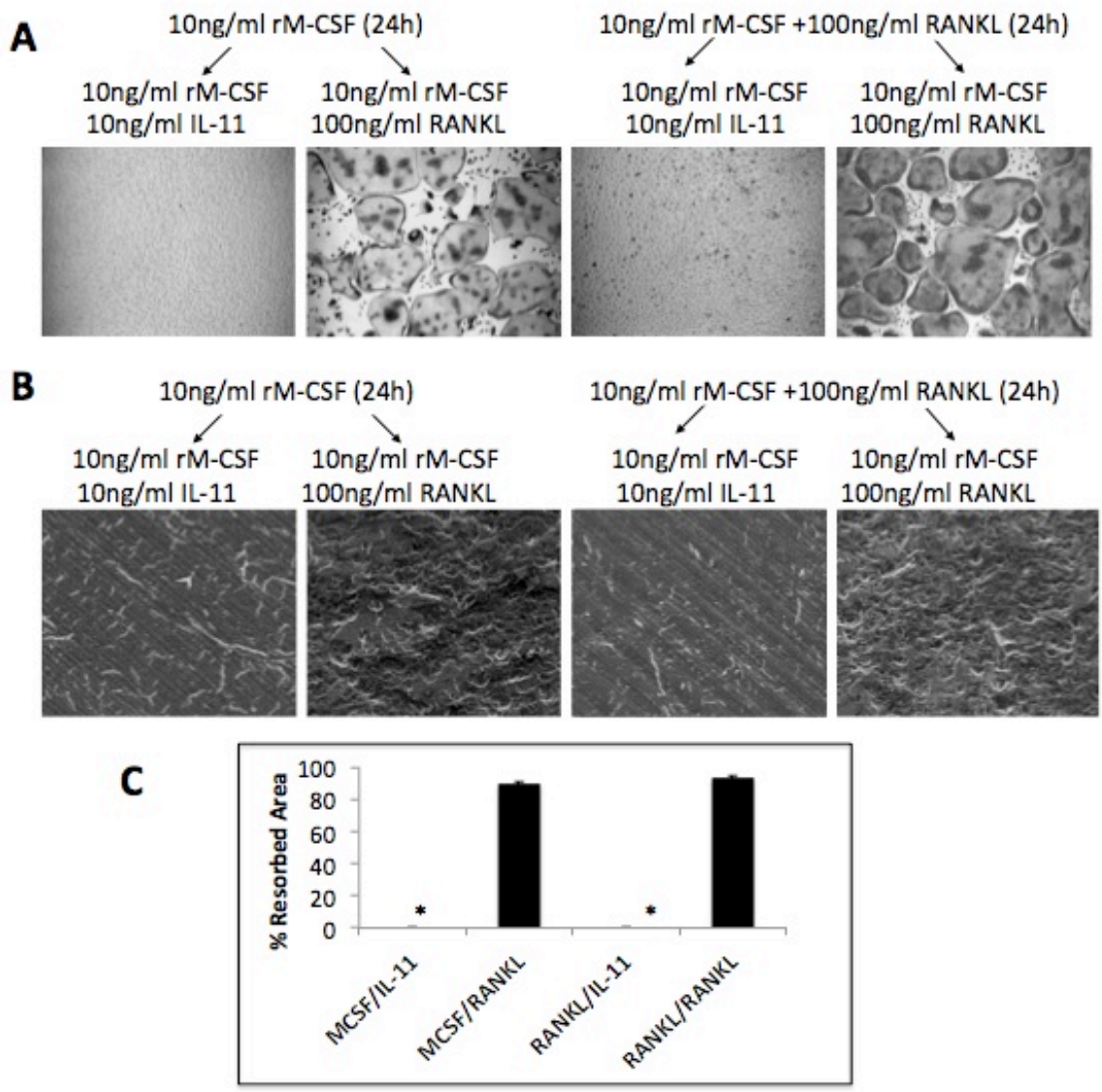

Figure 7 IL-11 fails to stimulate osteoclastogenesis even when BMMs are primed with RANKL for 24 hours. (A) BMMs were pretreated with rM-CSF (10ng/ml) or rM-CSF (10 ng/ml) and RANKL (100 ng/ml) for 24 hours and then cultured with rM-CSF (10 ng/ml) plus IL-11 (10 ng/ml) or RANKL (100 ng/ml) for 4 days in tissue culture dish. The cultures were then stained for TRAP activity. Each condition had three replicates (wells) and was repeated 4 times. A representative area of the culture from each condition is shown. (B) BMMS on bone slices were treated with the same conditions, but cultured for 9 days. Resorption pits were then visualized by SEM. Magnification by SEM was 200x. Each resorption assay had two replicates (bone slices). (C) Quantification of the bone resorption assays is shown, bars shown averaged ± S.E. *, $p<0.0001$.